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ATP receptors in hypothalamic neurons and pituitary cells : a novel mediator in the neuroendocrine system.

ATP RECEPTORS IN HYPOTHALAMIC NEURONS AND PITUITARY CELLS: A NOVEL MEDIATOR IN THE NEUROENDOCRINE SYSTEM

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ABSTRACT

In this thesis I have employed multiple *in vitro* models at both cellular and molecular levels to investigate a possible transmitter role for extracellular ATP in the neuroendocrine system.

ATP induced a rapid increase in $[Ca^{2+}]_i$ in a subset of cultured rat hypothalamic neurons. This intracellular Ca^{2+} response was highly specific and mediated by ATP receptors of the P_{2X} subtype, activation of which was independent of ATP hydrolysis and resulted in influx of Ca^{2+} largely through 'L' type voltage-gated Ca^{2+} channels.

ATP also caused intracellular Ca^{2+} responses in approximately 30% of rat pituitary cells in primary culture. The studies have firmly established the gonadotrope as one of direct target cells for ATP via a single class of receptor characterized as the P_{2U} subtype of ATP receptor. Activation of this receptor had no apparent effects on the cAMP and cGMP signalling systems but produced a biphasic cytosolic Ca^{2+} increase by mobilizing intracellular Ca^{2+} from gonadotropin releasing hormone- and thapsigargin-sensitive Ca^{2+} pools and stimulating Ca^{2+} influx through 'L' type voltage-sensitive Ca^{2+} channels. This Ca^{2+} response was mediated by a pertussis toxin-insensitive and phospholipase C-coupled G-protein. Agonist occupancy of this receptor caused translocation of protein kinase C (of the isozyme ε in αT3-1 cells) and a significant release of luteinizing hormone from superfused rat pituitary cells.

The present data have further revealed that substantial amount of ATP can be exocytotically released from pituitary cells, implying a possible paracrine and/or autocrine mechanism by which the extracellular nucleotides may exert their effects on pituitary cells.

In conclusion, this thesis provides strong evidence for a novel mediator role for ATP receptors in the neuroendocrine - and particularly in gonadotrope - function.

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Dedicated to my wife and my parents

DECLARATION

This is to declare that all the work contained in this dissertation was my own work and the views expressed in the dissertation are those of the author and not of the University.

Declared by Zhen-Ping Chen

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PUBLICATIONS RESULTING FROM THIS STUDY

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CONTENTS

ABBREVIATIONS

ApxA	adenine dinucleotide polyphosphates
BSA	bovine serum albumin
Ca^{2+}_i	intracellular Ca^{2+}
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
CNP	C-type natriuretic peptide
CRH	corticotropin-releasing hormone
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DTT	DL-dithiothreitol
ECL	enhanced chemiluminescence
FCS	fetal calf serum
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
GRH	growth hormone releasing hormone
HBSS	Hanks' balanced salt solution
HVA	high voltage-activated
IBMX	3-isobutyl-1-methylxanthine
IP	inositol phosphate
LH	luteinizing hormone
MAP1	microtubule-associated protein 1
NADPH	β-nicotinamide-adenine dinucleotide phosphate
NSE	neuron specific enolase
PACAP38	pituitary adenylate cyclase-activating polypeptide 38
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PKC	protein kinase C
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid
PTX	pertussis toxin
RIA	radioimmunoassay
RT-PCR	reverse transcription and polymerase chain reaction
SON	supraoptic nucleus
TRH	thyrotropin-releasing hormone

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PREFACE

This dissertation describes studies which were designed to examine whether extracellular ATP could play a special role as a transmitter in hypothalamic neurons and pituitary cells. The subject was initiated by the suggestion that extracellular ATP, acting on ATP receptors (P_2 purinoceptors), could be a neurotransmitter in both peripheral and central nervous systems.

It is just within the last few years that information about ATP receptors has dramatically expanded and this has led to the field being widely recognized. An overview of our current understanding of ATP receptors is, therefore, provided first in this dissertation to serve as an introduction to the field. What has been gained from my studies is then summarized in such way that each piece of work is presented with its own integrity including rationale, methods, results and discussion. For simplicity, the general details of all methods used are given in one section (Chapter 2).

Chapter 1. AN OVERVIEW OF ATP RECEPTORS

In 1929 Drury and Szent-Gyorgyi (1929) published the first report that adenylic acid and adenosine produced sinus bradycardia, complete atrioventricular block, a negative inotropic effect on the atrium and cessation of atrial fibrillation in the mammalian heart. In 1933 Gillespie (1933) found that ATP had an inotropic effect in frogs and Drury in 1936 suggested that ATP and adenosine might have different effects on cardiac contraction. Holton et al in 1950s demonstrated that ATP was present in dry powders of spinal roots (Holton & Holton 1954) and in the perfusate of the rabbit ear artery following nerve stimulation (Holton 1959), hinting a possible neurotransmitter role for ATP. It was almost a decade, however, before Burnstock and his colleagues observed a non-adrenergic and non-cholinergic element in the autonomic nervous system, and made the landmark proposals that ATP acted as a neurotransmitter in the peripheral autonomic nerves (Burnstock *et al.* 1970, Burnstock 1972, Burnstock *et al.* 1972, Burnstock 1976). This finding was confirmed and extended over the subsequent two decades, with ATP-evoked synaptic potentials being recorded between neurons in the central and peripheral systems (Edwards *et al.* 1992, Evans *et al.* 1992, Silinsky *et al.* 1992). The very recent molecular cloning of P_2 purinoceptors removed the last doubts about nucleotides being true extracellular mediators (Lustig *et al.* 1993, Webb *et al.* 1993, Brake *et al.* 1994, Valera *et al.* 1994), and it is now clear that ATP is a ubiquitous extracellular mediator acting on a superfamily of P_2 purinoceptors that may play important physiological and pathophysiological roles in a variety of biological processes including neurotransmission, platelet aggregation, muscle contraction and relaxation, secretion of insulin and surfactant, immune response and cell growth.

This review will mainly focus on the most recent advancements in this rapidly expanding field with an emphasis on ATP receptors in the central nervous system.

1.1 P_2 Purinoceptor Family

The receptors for purine compounds, purinergic receptors, constitute a large and diverse family that are expressed in a very wide range of tissues. In 1978, Burnstock proposed a basis for two types of purinergic receptor and subgrouped into P_1 and P_2 purinoceptors. P_1 receptors were preferentially activated by adenosine, while P_2 by ATP. This classification was widely adopted and extended. Adenosine receptors (P_1 purinoceptors) have been further classified into A_1, A_2 and A_3 (Fredholm *et al.* 1994). Correspondingly, ATP receptors (P_2 purinoceptors) were subdivided in 1985 by Burnstock and Kennedy into P_{2X} and P_{2Y} on the basis of rank-order of agonist's potency:- for P_{2X}, this was α,β-methylene ATP ≥ β,γ-methylene ATP > ATP = ADP > 2-methylthioATP with selective desensitization by α,β-methylene ATP; and for P_{2Y}, 2-methylthioATP >> ATP >> α,β-methylene ATP = β,γ-methylene ATP. This proposal was later found to correlate with receptor signal transduction mechanisms:- the P_{2X} being ligand-gated ion channels and the P_{2Y} G protein-coupled receptors. In 1986, Gordon added two additional P_2 purinoceptors into this scheme since the nucleotide receptors in platelets and immune cells did not fit very well into either the P_{2X} or P_{2Y} subtype. In platelets, ADP is the most potent agonist and ATP acts as an antagonist (termed P_{2T}), while in immune cells the most potent agonist is the fully ionized form of ATP (ATP^{4-}) (termed P_{2Z}). In some cell types, UTP has been found to be equipotent or even more potent than ATP, and the corresponding receptor was tentatively termed "nucleotide receptor" (Davidson *et al.* 1990) (which was consequently adopted by O'Connor *et al.* (1991) or re-termed P_{2U} receptor (Dubyak 1991). There is also evidence for purinoceptors that preferentially respond to adenine dinucleotide polyphosphates (ApxA) - P_{2D} receptors (Hilderman *et al.* 1991, Castro *et al.* 1992). An overview of P_2 purinoceptor is shown in Table 1-1.

P_{2X} purinoceptors

P_{2X} purinoceptors are ligand-gated intrinsic ion channels (Burnstock 1991, Bean 1992,

Table 1-1. Overview of P_2 Purinoceptors

P_2 receptor	P_{2X}		P_{2Y}	P_{2U}	P_{2T}	P_{2D}	P_{2Z}
main endogenous agonist	ATP		ATP	ATP/UTP	ADP	Apx	ATP^{4-}
structure / signal transduction	ligand-gated ion channels		G-protein coupled receptors				ion channels membrane pores?
agonist profile	2-meSATP ≥ ATP >>> α,β -meATP	α,β -meATP ≥ ATP, 2-meSATP	2-meSATP ≥ ATP >> α,β -meATP	UTP ≥ ATP	ADP	Apx	ATP^{4-}
reported antagonis	suramin reactive blue-2 trypan blue	suramin PPADS	suramin reactive blue-2	suramin	ATP FPL 66096 suramin		oxidized ATP
clone name	P_{2X} R1	P_{2X} receptor	P_{2Y1}	P2R, (HP2U)			
source	rat PC12 cells	rat vas deferens	chick brain	NG108-15, (CF/T43 & HT29)			
amino acid	472	399	362	373, (375)			
distribution	neuron, muscle		wide	wide	platelets leukaemia cell osteoblasts, BCEC	wide	immune cells

Notes: 1) 2-meSATP (2-methylthio ATP); α,β -meATP (α,β-methylene ATP); PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid); FPL 66096 (2-propylthio-D-β,γ-difluoromethylene ATP) and ApxA (diadenosine polyphosphates, mainly Ap3A, Ap4A, Ap5A and Ap6A).

2) NG108-15 (mouse neuroblastoma x rat C6 glioma cells); CF/T43 (a human airway epithelial cell line); HT29 (a human colonic epithelial cell line) and BCEC (brain capillary endothelial cells).

Edwards & Gibb 1993). The channels in the different cell types appear to be similar but not identical in their ligand selectivity and their ion permeability, indicating the existence of multiple subtypes. This has been clearly demonstrated by the recent cloning of two distinct ATP-gated ionotropic receptors (Valera *et al.* 1994, Brake *et al.* 1994).

Although P_{2X} purinoceptors have been reported to be present in lacrimal cells (Sasaki & Gallacher 1992, Vincent 1992) and microglial cells (Nörenberg *et al.* 1994, Langosch *et al.* 1994), they are predominantly confined to muscles and neurons. The former includes cardiac muscle (Friel & Bean 1988, Danziger *et al.* 1988, Scamps & Vassort 1990, Alvarez *et al.* 1990, Christie *et al.* 1992), skeletal muscle (Kolb & Wakelam 1983, Thomas & Hume 1990, Thomas *et al.* 1991) and smooth muscle such as vascular (Benham *et al.* 1987, Benham & Tsien 1987, Burnstock & Warland 1987, Ziganshin *et al.* 1994), bladder (Moss & Burnstock 1985, Inoue & Brading 1990, Schneider *et al.* 1991, Ziganshin *et al.* 1993) and vas deferens (Friel 1988, Bo *et al.* 1992). The latter includes both peripheral nerve cells such as sensory neurons (Krishtal *et al.* 1983, Bean 1990), dorsal horn neurons (Jahr & Jessell 1983), celiac neurons (Evans *et al.* 1992, Silinsky *et al.* 1992), parasympathetic neurons (Fieber & Adams 1991) and intracardiac neurons (Allen & Burnstock 1990) and central neurons (described later in this chapter).

P_{2Y} purinoceptors

P_{2Y} purinoceptors belong to G protein-coupled membrane receptors, activation of which causes an increase in phospholipid turnover and intracellular Ca^{2+} (Ca^{2+}_i) mobilization, presumably resulted from activation of phospholipase C (PLC). Burnstock's original classification criteria based on the agonist profile of 2-methylthioATP > ATP >> α,β-methylene ATP, β,γ-methylene ATP is still widely used for definition of this receptor (Burnstock & Kennedy 1985). P_{2Y} receptors have a wide tissue distribution and has been found in erythrocytes (Berrie *et al.* 1989, Boyer *et al.* 1989), leukemic basophils (Osipchuk & Cahalan 1992), pancreatic β-cells (Gylfe &

Hellman 1987), endothelial cells (Boeynaems & Pearson 1990), hepatocytes (Dixon *et al.* 1990, Keppens 1993), astrocytes (Pearce *et al.* 1989) and brain tissue (Webb *et al.* 1993, von Kügelgen *et al.* 1994).

P_{2U} purinoceptors

P_{2U} purinoceptors also belong to G protein-coupled membrane receptors and have a wide tissue distribution as does the P_{2Y} purinoceptor. Cells having been reported to possess P_{2U} receptors include neutrophils (Axtel *et al.* 1990, Kuroki & Minakami 1989), monocytes (Cowen *et al.* 1989), macrophages (Greenberg *et al.* 1988, Pfeilschifter *et al.* 1989), HL-60 promyelocytes (Dubyak *et al.* 1988, Stutchfield & Cockcroft 1990), osteoblast (Reimer & Dixon 1992), keratinocytes (Pillai & Bikle 1992), pituitary cells (Davidson *et al.* 1990, Chen *et al.* 1994b, Carew *et al.* 1994), FRTL-5 thyroid (Okajima *et al.* 1989, Sato *et al.* 1992), endothelial cells (Lustig *et al.* 1992, Purkiss *et al.* 1993), fibroblasts (Fine *et al.* 1989), hepatocytes (Dixon *et al.* 1990, Keppens 1993), PC12 cells (Murrin & Boarder 1992), chromaffin cell (Kim *et al.* 1990), airway epithelial cells (Brown *et al.* 1991, Parr *et al.* 1994), mesangial cells (Pfeilschifter 1990), amnion cells (Vander Kooy *et al.* 1989), aortic vascular muscle (Tawada *et al.* 1987) and Erhlich ascites tumor cells (Dubyak 1986).

There is some evidence to suggest that UTP and ATP act on different receptors. For example, in perfused rat liver, ATP and UTP produced differential actions on portal pressure, glucose output, K^+ uptake and Ca^{2+} release (Häussinger *et al.* 1987). Differentiation of HL60 cells altered the response to UTP but not ATP (Stutchfield & Cockcroft 1990). α,β-methylene ATP desensitized more to ATP than to UTP in vasoconstriction of rabbit ear artery (von Kügelgen *et al.* 1987). Pertussis toxin (PTX) and adenylate cyclase activators blocked the activation of β-nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase by UTP but not by ATP in human neutrophil and HL-60 leukaemic cells (Seifert *et al.* 1989). Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) selectively antagonized α,β-methylene ATP- but not

UTP-evoked depolarization in the rat superior cervical ganglion (Connolly 1994). In contrast, many other cells or tissues have been shown to exhibit cross desensitization between ATP and UTP to suggest the responses are mediated by a common nucleotide receptor that recognizes both ATP and UTP (Pfeilschifter 1990, Brown *et al.* 1991, Iredale *et al.* 1992a, Chen *et al.* 1994c), perhaps at distinct binding sites (Erb *et al.* 1993).

P_{2D} purinoceptors

The existence of P_{2D} purinoceptors have been demonstrated by specific binding sites for [^{3}H]-Ap4A in cultured chromaffin cells (Pintor *et al.* 1991) and rat brain synaptosomes (Pintor *et al.* 1993). This is strengthened by the studies using a monoclonal antibody (against a putative Ap4A receptor) which bound to the mouse heart cell surface and inhibited the [^{3}H]-Ap4A binding (Walker *et al.* 1993). To date, it appears that all diadenosine polyphosphates (Ap3A, Ap4A, Ap5A and Ap6A) act on a single class membrane receptors (Pintor *et al.* 1991, Hilderman *et al.* 1994) and that the corresponding binding sites are found in heart, fat, brain, muscle, liver, spleen and kidney (Hilderman *et al.* 1991). Some cells such as cortical neurons (Stone & Perkins 1981), brain synaptosomes (Pintor *et al.* 1993), chromaffin cells (Pintor *et al.* 1991), heart cells (Walker *et al.* 1993) and platelets (Zamecnik *et al.* 1992) have been reported as direct targets for adenine dinucleotide polyphosphates.

P_{2T} purinoceptors

P_{2T} purinoceptors are thought to be confined to platelets (Gordon 1986) but recent evidence seems to suggest that they exist elsewhere, such as in brain capillary endothelial cells (Frelin *et al.* 1993, Vigne *et al.* 1994), K562 leukaemia cells (Murgo & Sistare 1992), megakaryocytic Dami cells (a human megakaryocytic leukaemia cell line) (Murgo *et al.* 1994) and UMR-106 osteoblasts (Sistare *et al.* 1994).

P_{2Z} purinoceptors

P_{2Z} purinoceptors appear to be expressed exclusively in immune cells, though the pharmacological data of P_2 purinoceptor in rat parotid acinar cells (McMillian *et al.* 1988, Tenneti & Talamo 1993) are also indicative of the P_{2Z} subtype. The structure of P_{2Z} purinoceptor is poorly understood. Some evidence indicates that the P_{2Z} receptors in macrophages are coupled to a cation channel, in addition to being non-selective membrane pores (permeable to molecules with Mr up to about 900 and its formation being both time- and temperature-dependent) (Nuttle & Dubyak 1994). It has even been suggested that the gap junction protein connexin-43 in mouse macrophages is the P_{2Z} receptor on the basis that the macrophage expresses the connexin-43 mRNA/protein and that the property of ATP-induced pores is similar to that for gap junctions (Beyer & Steinberg 1991).

Other nucleotide receptors

Recent data appear to indicate the existence of novel groups of receptors for guanine and uridine compounds, respectively. In rat mesenteric artery, GTP and guanosine (10 μM - 1 mM) induced both endothelium-dependent and -independent relaxations, which were not affected by 8-phenyltheophylline and reactive blue 2 (Vuorinen *et al.* 1994). PLC in the rat glioma cell line C6-2B was activated by UTP or UDP but not by ATP, ADP and other analogues (Lazarowski & Harden 1994).

Re-classification of P_2 receptors

A new P_2 purinoceptor subclassification scheme rather different from the above-described current classification has recently been proposed by Abbracchio and Burnstock (Abbracchio & Burnstock 1994). In this new scheme, P_2 purinoceptors are divided into three groups:- $P2X_1$ - $P2X_4$ for ligand-gated ion channels (Table 1-2), $P2Y_1$ - $P2Y_7$ for all G protein-coupled P_2 purinoceptors (Table 1-3), and P2Z for

Table 1-2. Abbracchio and Burnstock's proposed new subclassification of P2X-ion gated purinoceptor family

Proposed subtype	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$
Selective agonists	2-(4-nitrophenylethylthio)ATP > 3'-amino-3'-deoxy-ATP = 2-hexylthioATP = 2-cyclo-hexylthioATP	5-fluoro-UTP > 2-hexylthioATP = 3'-acetylamino-3'-deoxy-ATP > 3'(4-hydroxyphenylproprionyl-amino)-3'-deoxy-ATP	No selective agonists available but the compounds selective on the other subtypes are inactive	No selective agonists available
Examples of tissue	guinea-pig vas deferens	rat bladder	vascular smooth muscle	neurons

Table 1-3. Abbracchio and Burnstock's proposed new subclassification of P2Y-G-protein linked purinoceptor family

Proposed subtype	$P2Y_1$	$P2Y_2$	$P2Y_3$	$P2Y_4$	$P2Y_5$	$P2Y_6$	$P2Y_7$
Formerly name		P2U	P2T				P2D
Agonist potency	2meSATP ≥ ATP >> ADP >> αβmeATP	ATP ≥ UTP = ATPγS	2meSADP > ADP	2meSATP >> ATP = ADP = αβmeATP >> βγmeATP	2meSATP ≥ ATP = ADP >> αβmeATP βγmeATP inactive	2meSATP > ATP > ADP?	diadenosine poly-phosphates
Selective agonists	2meSATP	UTPγS		2'-deoxy-ATP and also N6-methyl-ATP selective for taenia coli	8-(6-aminohexyl-amino)-ATP and ATP-N-oxide selective for endothelial cells	No selective agonists available but the compounds selective for P2Y4 and P2Y5 are inactiveon vascular smooth muscle	
Clone	Clone 803 (Webb 1993)	pP2R (Lustig 1993) HP2U (Parr 1994)					
Examples of Tissue	chick brain	rat renal mesangial cell	platelets	Turkey erythrocytes	rabbit aorta	rabbit coronary arteries	rat brain synapto-somes

nonselective pore-forming receptors.

1.2 Signal Transduction

The main signal transduction pathways involved in the actions of extracellular nucleotides are briefly summarized in Figure 1-1.

P_{2X} purinoceptor - ligand-gated ion channels

Activation of P_{2X} purinoceptors leads to the opening of ion channels. Most reported ATP-activated ion channels, including the two cloned P_{2X} receptors, have been shown to be non-selective cation (K^+, Na^+, Ca^{2+}) ion channels, but in developing chick skeletal muscle a single class of ATP-activated ion channels was found to be permeable to both cations (K^+, Na^+, Ca^{2+}) and anions (Cl^-, NO_3^-) (Thomas & Hume 1990).

$P_{2Y/2U/2T/2D}$ purinoceptors - G protein-coupled membrane receptors

P_{2Y}, P_{2U}, P_{2T} and P_{2D} purinoceptors form a distinct large receptor family and are all coupled to G-proteins (Webb *et al.* 1993, Lustig *et al.* 1993, Murgo *et al.* 1994, Gasmi *et al.* 1994, Castro *et al.* 1994). P_{2Y}, P_{2U} and P_{2D} in most cases are predominantly linked to PLC, resulting in an increase in phospholipid turnover, intracellular Ca^{2+} mobilization and formation of diacylglycerol (DAG) (Berridge 1993).

In addition to the inositol phosphate (IP) and Ca^{2+} signalling, other G protein-linked signal pathways are also involved in signal transduction from P_2 receptors, which may be secondary to activation of the PLC/IP/Ca^{2+}/DAG system. For example, increased intracellular Ca^{2+} can exert its effects on adenylate cyclase in a inhibitory (e.g.: in pituitary cells and cardiac cells) or stimulatory fashion via the complex of Ca^{2+}-calmodulin (e.g.: in neurons). A Ca^{2+}-insensitive adenylate cyclase also exists (Cooper

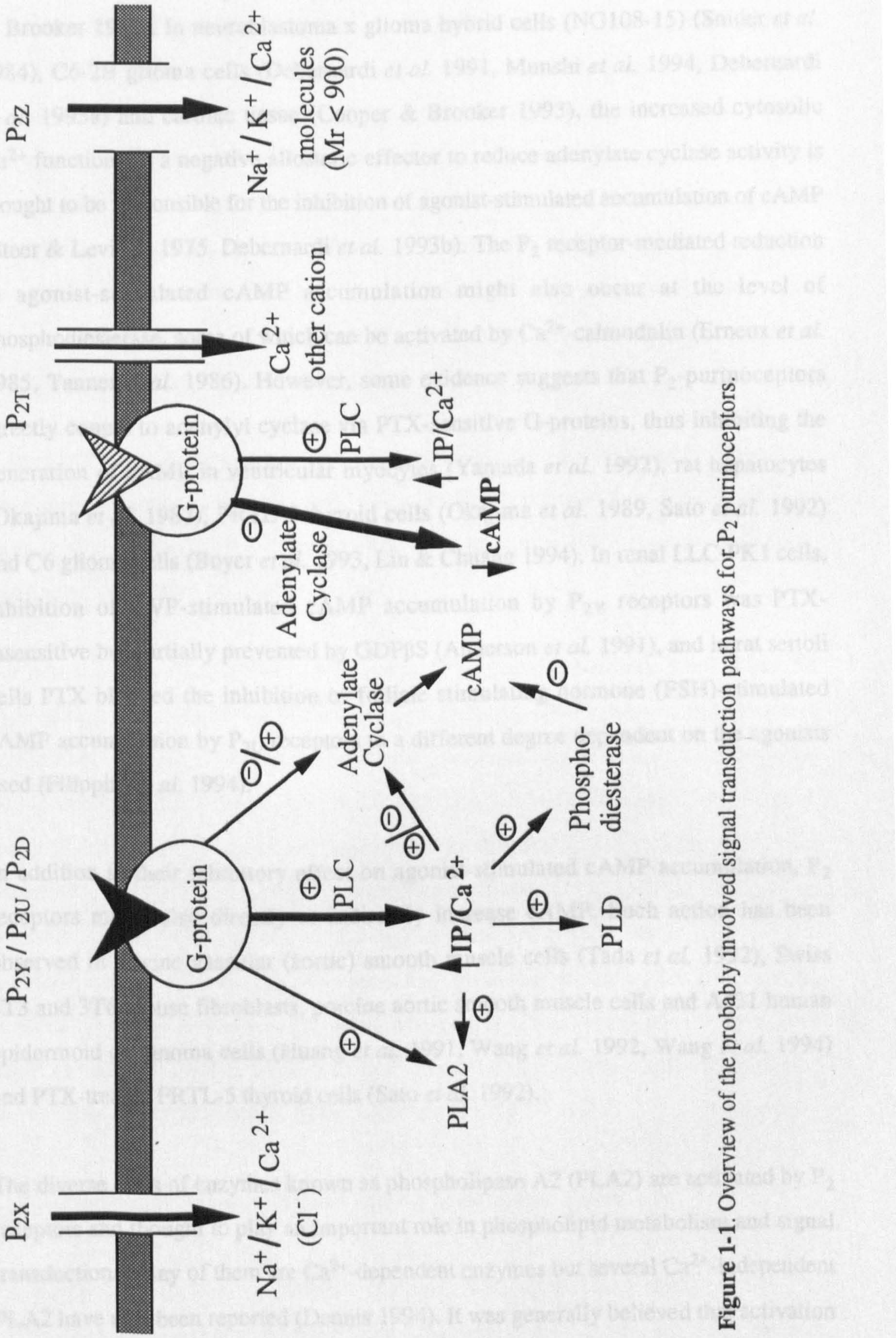


Figure 1-1. Overview of the probably involved signal transduction pathways for P_2 purinoceptors.

& Brooker 1993). In neuroblastoma x glioma hybrid cells (NG108-15) (Snider *et al.* 1984), C6-2B glioma cells (Debernardi *et al.* 1991, Munshi *et al.* 1994, Debernardi *et al.* 1993a) and cardiac tissue (Cooper & Brooker 1993), the increased cytosolic Ca^{2+} functions as a negative allosteric effector to reduce adenylate cyclase activity is thought to be responsible for the inhibition of agonist-stimulated accumulation of cAMP (Steer & Levitzki 1975, Debernardi *et al.* 1993b). The P_2 receptor-mediated reduction in agonist-stimulated cAMP accumulation might also occur at the level of phosphodiesterase, some of which can be activated by Ca^{2+}-calmodulin (Erneux *et al.* 1985, Tanner *et al.* 1986). However, some evidence suggests that P_2-purinoceptors directly couple to adenylyl cyclase via PTX-sensitive G-proteins, thus inhibiting the generation of cAMP in ventricular myocytes (Yamada *et al.* 1992), rat hepatocytes (Okajima *et al.* 1987), FRTL-5 thyroid cells (Okajima *et al.* 1989, Sato *et al.* 1992) and C6 glioma cells (Boyer *et al.* 1993, Lin & Chuang 1994). In renal LLC-PK1 cells, inhibition of AVP-stimulated cAMP accumulation by P_{2Y} receptors was PTX-insensitive but partially prevented by GDPβS (Anderson *et al.* 1991), and in rat sertoli cells PTX blocked the inhibition of follicle stimulating hormone (FSH)-stimulated cAMP accumulation by P_{2U} receptors to a different degree dependent on the agonists used (Filippini *et al.* 1994).

In addition to their inhibitory effect on agonist-stimulated cAMP accumulation, P_2 receptors might also directly or indirectly increase cAMP. Such action has been observed in bovine vascular (aortic) smooth muscle cells (Tada *et al.* 1992), Swiss 3T3 and 3T6 mouse fibroblasts, porcine aortic smooth muscle cells and A431 human epidermoid carcinoma cells (Huang *et al.* 1991, Wang *et al.* 1992, Wang *et al.* 1994) and PTX-treated FRTL-5 thyroid cells (Sato *et al.* 1992).

The diverse class of enzymes known as phospholipase A2 (PLA2) are activated by P_2 receptors and thought to play an important role in phospholipid metabolism and signal transduction. Many of them are Ca^{2+}-dependent enzymes but several Ca^{2+}-independent PLA2 have also been reported (Dennis 1994). It was generally believed that activation

of PLA2 and arachidonic acid release by P_2 receptors was downstream of PLC/IP/Ca^{2+}. However, in 3T3, 3T6, and A431 cells (Huang *et al.* 1991) and in porcine aortic smooth muscle cells (Wang *et al.* 1992) PTX blocked P_2 receptor-activated arachidonic acid release and prostaglandin synthesis, suggesting that P_2 receptors may directly couple to PLA2 via PTX-sensitive G proteins. This suggestion was supported by recent genetic findings that a mutation of $G_{\alpha i2}$ subunit with single amino acid change from Glycine(203) to Threonine inhibited thrombin and ATP receptor stimulation of arachidonic acid release independent of adenylate cyclase inhibition and Ca^{2+} mobilization (Winitz *et al.* 1994).

In some cell types, phospholipase D (PLD) and cGMP are also involved in P_2 receptor signal transduction, and activation of PLD and cGMP generating system appear to be dependent on the PLC/IP/Ca^{2+} system (Martin & Michaelis 1989, Purkiss *et al.* 1993, Gustavsson *et al.* 1993, Snider *et al.* 1984).

The signalling mechanism for P_{2T} receptors is slightly different from those for $P_{2Y}/P_{2U}/P_{2D}$. Either inhibition of the adenylate cyclase/cAMP system or stimulation of the PLC/IP/Ca^{2+} system or both are employed as the main signal transduction pathways for P_{2T} receptors in platelet (Cooper & Rodbell 1979), K562 leukaemia cells (Murgo & Sistare 1992), megakaryocytic Dami cells (a human megakaryocytic leukaemia cell line) (Murgo *et al.* 1994) and UMR-106 osteoblasts (Sistare *et al.* 1994). Recently an atypical P_{2T} receptor was found in brain capillary endothelial cells, where ADP induced the mobilization of a thapsigargin-sensitive intracellular Ca^{2+} pool in a manner independent of the formation of inositol phosphates (Frelin *et al.* 1993, Vigne *et al.* 1994). In addition, there is some evidence to suggest that ADP activates ligand-gated ion channels in human platelets (Mahautsmith *et al.* 1990).

P_{2Z} purinoceptor - ion channels and/or membrane pores

The signal transduction mechanism is poorly understood. Activation of the P_{2Z} purinoceptor increases plasma membrane permeability by opening ion channels (for

cation influx such as Na^+, K^+ and Ca^{2+}) and/or by formation of membrane pores (permeable to molecules with Mr up to about 900 such as N-methyl-D-glucamine (NMG+), ethidium+ and fura-2) (Nuttle & Dubyak 1994, Chen *et al.* 1994a).

1.3 Molecular Biology of P_2 Purinoceptors

The molecular cloning of two ATP-gated ion channel receptors and two G protein-linked ATP receptors are exciting and very recent developments in the field.

ATP-gated ion channels

Two cDNAs encoding for putative P_{2X} receptors were independently isolated from vas deferens and PC12 cells, and represent two distinct subtypes of intrinsic non-selective cation ion channels (Valera *et al.* 1994, Brake *et al.* 1994). Surprisingly, the pharmacological agonist profiles for these two clones do not fit well into the widely used P_{2X} purinoceptor agonist potency criteria (i.e. α,β-methylene ATP > β,γ-methylene ATP > ATP, 2-methylthioATP) (Burnstock & Kennedy 1985). The putative P_{2X}R1 has 472 amino acids with a predicted mass of 52.6 KDa (Brake *et al.* 1994), consistent with the findings of a 50 to 53 KDa membrane protein labelled with [^{32}P]BzATP (3'-O-4-benzoylbenzoyl ATP) in PC12 cells (Majid *et al.* 1992, Rhoads *et al.* 1993), suggesting that the P_{2X} receptor, at least in PC12 cells, is not glycosylated. The cloned, ionotropic ATP receptor from PC12 cells (P_{2X}R1) (Brake *et al.* 1994), when expressed in *Xenopus* oocytes, showed the following rank-order of agonist potency in production of inward currents: ATP = 2-methylthioATP >>> α,β-methylene ATP and β,γ-methylene ATP. This agonist profile resembles those previously found for ionotropic ATP receptors in PC12 cells (Nakazawa *et al.* 1990a, Majid *et al.* 1992, Rhoads *et al.* 1993), rat parasympathetic cardiac ganglia (Fieber & Adams 1991), in rat sympathetic neurons (Cloues *et al.* 1993), neurons of guinea pig submucous plexus (Barajas-Lopez *et al.* 1994), guinea-pig intracardiac neurons (Allen & Burnstock

1990), rat nucleus solitarii neurons (Ueno *et al.* 1992a) and rat microglial cells (Nörenberg *et al.* 1994, Langosch *et al.* 1994).

Simultaneously, another ionotropic ATP receptor was cloned from rat vas deferens by Valera, et al (Valera *et al.* 1994). This cDNA, when transiently expressed in human embryonic kidney HEK293 cells, exhibited the following agonist potency in evoking inward currents: 2-methylthioATP ≥ ATP > α,β-methylene ATP > ADP, closely resembling those observed in rabbit ear artery smooth muscle (Benham *et al.* 1987, Benham & Tsien 1987) and rat locus coeruleus neurons (Tschöpl *et al.* 1992, Shen & North 1993). This putative P_{2X} receptor has 399 amino acids with a predicted mass of 45 KDa, apparently smaller than the reported P_{2X} receptor of 62 KDa solubilized from rat vas deferens (Bo *et al.* 1992). These two molecules might be identical - the higher mass of 62 KDa due to glycosylation, or representative of two different proteins since there are two [^{3}H] α,β-methylene ATP binding sites detected in rat vas deferens (Bo *et al.* 1992).

Both cloned ATP-gated ionotropic receptors share around 40% amino acid sequence identity (Figure 1-2). Their overall structures are very similar:- containing two hydrophobic putative transmembrane domains (about 20 and 28 amino acids) linked by a large extracellular hydrophilic cysteine-rich domain (about 280 amino acids) to both N- and C-terminals, which are intracellular. Both receptors bear no apparent sequence identity to any other receptor proteins and it appears that ATP-gated intrinsic ion channels form their own distinct family. Strikingly, both receptors have approximately 45% sequence identity with RP-2, a partial cDNA clone isolated from rat apoptotic thymocytes (Owens *et al.* 1991). The implication for such high sequence similarity is unknown at present.

The tissue distribution for these two putative P_{2X} receptors are very wide. Transcripts have been detected in brain, spinal cord, the superior ganglion, intestine, vas deferens, bladder, adrenal gland, pituitary and testis but not in heart, kidney, liver, ovary, lung,

PC12	MVRRLARGCW	SAFWDYETPK	VIVVRNRRLG	FVHRMVQLLI	LLYFV.WYVF	IVQKSYQDSE	59
vas D	MARRLQDELS	AFFFEYDTPR	MVLVRNKKVG	VIFRLIQLVV	LVYVIGW.VF	VYEKGYQTS.	58
					TM I		
PC12	TGPESSIITK	VKGITMS...	..EDKVWDVE	EYVKPPEGGS	VVSIITRIEV	TPSQTLGTCP	114
vas D	SDLISSVSVK	LKGLAVTQLQ	GLGPQVWDVA	DYVFPAHGDS	SFVVMTNFIV	TPQQTQGHCA	116
PC12	ESMRVHSSTC	HSDDDCIAGQ	LDMQGNGIRT	GHCVPYYHGD	SKTCEVSAWC	PVE.DGTSDN	173
vas D	EN..PEGGIC	QDDSGCTPGK	AERKAQGIRT	GNCVPF.NGT	VKTCEIFGWC	PVEVDDKIPS	175
PC12	HFLGKMAPNF	TILIKNSIHY	PKFKFSKGNI	ASQ.KSDYLK	HCTFDQDSDP	YCPIFRLGFI	232
vas D	PALLREAENF	TLFIKNSISF	PRFKVNRRNL	VEEVNGTYMK	KCLYHKIQHP	LCPVFNLGYV	235
PC12	VEKAGENFTE	LAHKGGVIGV	IINWNCDLDL	SESECNPKYS	FRRLDPKYDP	ASSGYNFRFA	292
vas D	VRESGQDFRS	LAEKGGVVGI	TIDWKCDLDW	HVRHCKPIYQ	FHGLYGE.KN	LSPGFNFRFA	294
PC12	KYYKINGTTT	TRTLIKAYGI	RIDVIVHGQA	GKFSLIPTII	NLATALTSIG	VGSFLCDWIL	352
vas D	RHF.VQNGTN	RRHLFKVFGI	HFDILVDGKA	GKFDIIPTMT	TIGSGIGIFG	VATVLCDLLL	353
					TM II		
PC12	LTFMNKNKLY	SHKKFDKVRT	PKHPSSRWPV	TLALVLGQIP	PPPSHYSQDQ	PPSPPSGEGP	412
vas D	LHILPKRHYY	KQKKFKYAED	MGPGEGEHDP	VATSSTLGLQ	ENMRTS		399
PC12	TLGEGAELPL	AVQSPRPCSI	SALTEQVVDT	LGQHMGQRPP	VPEPSQQDST	STDPKGLAQL	472

Figure 1-2. Deduced amino acid sequences of P_{2X} purinoceptors aligned for maximum homology. The approximate positions of the transmembrane domains (TM) are underlined. Data from Brake 1994 for a P_{2X} receptor isolated from PC12 cells and Valera 1994 for a P_{2X} receptor derived from rat vas deferens.

spleen and skeletal muscle by the PC12 cDNA in Northern blots or *in situ* hybridization (Brake *et al.* 1994). Transcripts corresponding to the rat vas deferens cDNA were detected in bladder, lung, spleen, spinal cord, coeliac ganglia, PC12, retina and thymus (Valera *et al.* 1994). The distribution obviously overlaps and this may reflect the presence of multiple subtypes of P_{2X} receptor in a given cell type or tissue, or may result from the high nucleic acid sequence similarity (> 50%) between these two clones.

G protein-coupled purinoceptors

A cDNA (P2R) isolated from mouse NG108-15 neuroblastoma x rat C6 glioma hybrid cells (Lustig *et al.* 1993) has been characterized to encode a functional P_{2U} receptor with the following agonist potency in activation of PLC when expressed in *Xenopus laevis* oocytes (Lustig *et al.* 1993) or K-562 human leukaemia cell line (Erb *et al.* 1993): ATP = UTP >> ADP, 2-methylthioATP and β,γ-methylene ATP. The cDNA gives rise to a 373 residue protein with a predicted mass of 42 KDa. Glycosylated, this cloned P_{2U} receptor protein expressed in K-562 human leukaemia cells occurs as a 53 KD membrane protein as determined by photoaffinity labelling with [^{32}P]BzATP (Erb *et al.* 1993). Interestingly, [^{32}P]BzATP labelling was inhibited by ATP but not UTP, suggesting that each nucleotides may have a distinct binding site on the receptor (Erb *et al.* 1993). A putative human P_{2U} receptor cDNA, isolated from an airway epithelial cell line (CF/T43) and a colonic epithelial cell line (HT-29) (Parr *et al.* 1994), has an 89% DNA sequence homology and functional similarities to the mouse neuroblastoma P_{2U} receptor.

A putative receptor cDNA (P_{2Y1}) was obtained from the embryonic chick brain and showed a agonist potency order characteristic of P_{2Y} receptors:- 2-methylthioATP ≥ ATP > ADP >>> UTP, α,β-methylene ATP and β,γ-methylene ATP in the production of slow inward currents (Webb *et al.* 1993). It has 362 amino acids with a predicted mass of 41 KDa.

P2Y1	MTEALISAAL	NGTQPELLAG	GWAAGNATTK	CSLTKTGFQF	YYLPTVYILV	FITGFLGNSV	60
P2U	MAAD	LEPWNSTING	TWEGDELGYK	CRF.NEDFKY	VLLPVSYGVV	CVLGLCLNVV	53
						TM I	
P2Y1	AIWMFVFHMR	PWSGISVYMF	NLALADFLYV	LTLPALIFYY	FNKTDWIFGD	VMCKLQRFIF	120
P2U	ALYIFLCRLK	TWNASTTYMF	HLAVSDSLYA	ASLPLLVYYY	ARGDHWPFST	VLCKLVRFLF	113
			TM II				
P2Y1	HVNLYGSILF	LTCISVHRYT	GVVHPLKSLG	RLKKKNAVYV	SSLVWALVVA	VIAPILFYSG	180
P2U	YTNLYCSILF	LTCISVHRCL	GVLRPLHSLR	WGRARYARRV	AAVVWVLVLA	CQAPVLYFVT	173
	TM III				*TM IV*		
P2Y1	TGVRRNKTIT	CYDTTADEYL	RSYFVYSMCT	TVFMFCIPFI	VILGCYGLIV	KALI...YKD	237
P2U	TSVRGTR.IT	CHDTSARELF	SHFVAYSSVM	LGLLFAVPFS	VILVCYVLMA	RRLLKPAYGT	232
					TM V		
P2Y1	LDNSP.LRRK	SIYLVIIVLT	VFAVSYLPFH	VMKTLNLRAR	LDFQTPQM.C	AFNDKVYATY	295
P2U	TGGLPRAKRK	SVRTIALVLA	VFALCFLPFH	VTRTLYY...	.SFRSLDLSC	HTLNAINMAY	288
		TM VI					
P2Y1	QVTRGLASLN	SCVDPILYFL	AGDTFRRRLS	RATRKSSRRS	EPNVQSKSEE	MTLNILTEYK	355
P2U	KITRPLASAN	SCLDPVLYFL	AGQRLVRFAR	DAKPPTEPTP	SPQARRKLGL	HRPNRTVRKD	348
	TM VII						
P2Y1	QNGDTSL						362
P2U	LSVSSDDSRR	TESTPAGSET	KDIRL				373

Figure 1-3. Deduced amino acid sequences of P_2 purinoceptors (P_{2U} and P_{2Y1}) aligned for maximum homology. The approximate positions of the transmembrane domains (TM) are underlined. Data from Webb 1993 for the P_{2Y1} receptor and from Lustig 1993 for the P_{2U} receptor.

Table 1-4. Amino acid homology comparison of the cloned P2Y1 and P2U purinoceptors with other known G-protein coupled receptors.

Receptor	Species	% Identity with P_{2Y1} (chick)	% Identity with P_{2U} (murine)
RDC1	canine	27	
angiotensin II type I	human / (bovine)	27	(22)
Thrombin	human	25	25
Platelet-activating factor	guinea-pig	25	25
C5a anaphylatoxin	human	23	
Neuromedin K	rat	23	
Interleukin 8	human / (rabbit)	22	(23)
Bradykinin B2	rat	22	
GPRN1 *	human		21
Neurotensin	rat	21	
Endothelin B	human	21	
Gastrin-releasing peptide	murine	21	
Adenosine A1	canine	21	
Substance P	human	20	
Neurokinin 2	human	20	
Adenosine A2	canine	18	
cAMP	slime mold	17	
Adenosine	unknown		< 12
cAMP	unknown		< 12

*: a putative vasoactive interstinal peptide receptor.

Figures in brackets correspond to the species in brackets. Data adapted from Lustig, 1993 and Webb, 1993.

These cloned receptors (P_{2U} and P_{2Y}) showed structural features characteristic of G protein-coupled receptors including seven hydrophobic transmembrane domains (α-helices), consensus sequences for N-linked glycosylation near the N-terminal and two conserved cysteine residues in the first two extracellular loops (Figure 1-3). They are among the smallest proteins (362 amino acids for the P_{2Y1} and 373 for the P2R) in the superfamily of G protein-coupled receptors and share approximately 40% amino acid sequence identity. The homology of both receptors to other members of the G protein-coupled receptor family is low (Table 1-4). Northern blotting shows a wide tissue distribution for these cloned receptors. Transcripts for P_{2Y1} were detected in the brain, spinal cord, gastro-intestinal tract, spleen and skeletal muscle, but not in heart, liver, stomach, lung and kidney (Webb *et al.* 1993), and the P2R was seen in the spleen, testis, kidney, liver, lung, heart and brain (Lustig *et al.* 1993).

1.4 ATP receptors in the CNS

Since Burnstock's first proposal that purinergic nerves, which release ATP as their primary neurotransmitter (Su *et al.* 1971), might mediate nonadrenergic and noncholinergic relaxation of gastrointestinal smooth muscle (Burnstock *et al.* 1970), a large body of evidence has accumulated to unambiguously demonstrate that ATP, released upon stimulation and acting on ATP receptors, is a neurotransmitter in the autonomic nervous system and peripheral sensory nerves (Burnstock 1972, Burnstock 1976, von Kügelgen & Starke 1985, Evans *et al.* 1992, Trezise *et al.* 1993, Krishtal *et al.* 1983, Krishtal *et al.* 1988, Bean 1990, Bean *et al.* 1990).

As in peripheral tissues, multiple ATP receptors (mainly P_{2X}, P_{2Y}, P_{2U} and P_{2D}) are expressed in the CNS (Valera *et al.* 1994, Brake *et al.* 1994, Lustig *et al.* 1993, Webb *et al.* 1993, Hilderman *et al.* 1991). P_{2X} receptors in the CNS are largely confined to neurons (Salt & Hill 1983, Jahr & Jessell 1983, Tschöpl *et al.* 1992, Harms *et al.* 1992, Ueno *et al.* 1992b, Edwards *et al.* 1992, Bean 1992, Shen &

North 1993) but also present in microglial cells (Nörenberg *et al.* 1994, Langosch *et al.* 1994). Binding experiments show that a widespread distribution of [^{3}H]α,β-methylene ATP binding sites (as an indication for P_{2X} receptors) in rat brain and spinal cord, and many structures in the CNS are densely labelled including thalamus, amygdaloid, substantia nigra, cerebral cortex, hypothalamus, caudate putamen, geniculate nuclei, medial habenula and the intermediate zone of grey matter in the spinal cord (Bo & Burnstock 1994, Michel & Humphrey 1993). Though the P_{2Y1} receptor was cloned from brain tissue and its transcripts were highly expressed in brain tissue (Webb *et al.* 1993), the available data seems to suggest that the G protein-linked P_2 receptors in the CNS are largely located in non-neural cells (Kastritsis *et al.* 1992, Bruner & Murphy 1993, Boyer *et al.* 1993, Munshi *et al.* 1994, Lin & Chuang 1994). However, a presynaptic P_{2Y} receptor (von Kügelgen *et al.* 1994) has been defined and P_{2U} receptor has also been found in N1E-115 neuroblastoma cell line (Iredale *et al.* 1992a), hinting that nucleotides may also be involved in slow synaptic transmission and metabotropic actions via G proteins.

Neurons

ATP-evoked excitatory responses have been observed in many types of central neurons:- these include cerebral cortical neurons (Phillis *et al.* 1975, Phillis *et al.* 1979), caudal trigeminal nucleus (Salt & Hill 1983), nucleus tractus solitarii neurons (Ueno *et al.* 1992b), rostral ventrolateral reticular nucleus of the medulla oblongata (into which the microinjection of ATP produced a powerful pressor response (Sun *et al.* 1992)), caudal medulla neurons (Day *et al.* 1993), locus coeruleus neurons (Harms *et al.* 1992, Tschöpl *et al.* 1992, Shen & North 1993), medial habenula neurons (Edwards *et al.* 1992), vestibular and auditory cells (Shigemoto & Ohmori 1990, Nakagawa *et al.* 1990, Dulon *et al.* 1991, Housley *et al.* 1992, Aubert *et al.* 1994, Nilles *et al.* 1994), hippocampal neurons (Wieraszko & Seyfried 1989, Inoue *et al.* 1992, Mironov 1994), thalamus neurons (Mironov 1994), hypothalamic neurons (Chen *et al.* 1994a), and neurons in the dorsal horn of spinal cord (Jahr & Jessell 1983, Fyffe & Perl 1984, Salter & Henry 1985, Salter & Hicks 1994).

In addition to acting on postsynaptic P_2 purinoceptors, ATP, co-released with noradrenaline or acetylcholine, also plays a feedback role in modulation of transmitter release via presynaptic P_2 purinoceptors in the autonomic nervous system. The inhibition is thought to be the main form of modulation in sympathetic neuro-effector transmission and has been seen in the mouse and rat isolated vas deferens (von Kügelgen *et al.* 1989, Kurz *et al.* 1993, von Kügelgen *et al.* 1994, Todorov *et al.* 1994) and chick sympathetic neurons in culture (Allgaier *et al.* 1994). The facilitation on noradrenergic and/or cholinergic transmission, however, has also been reported in the rabbit ear artery (Miyahara & Suzuki 1987), guinea pig ileum (Sperlagh & Vizi 1991), saphenous artery (Todorov *et al.* 1994) and rat sympathetic neurons (Boehm 1994). One can speculate that such ATP-mediated presynaptic modulation may be common to the CNS, particularly in noradrenergic and cholinergic pathways. This is supported by the finding that in rat cortical noradrenergic axons, a separate P_2-purinoceptor, in addition to the known adenosine A1-receptor, was identified (P_{2Y}-like subtype) and functioned as if it was in the peripheral sympathetic nervous system (von Kügelgen *et al.* 1994).

Glial cells

Glial cells, as a constitutional and functional part of the central nervous system, are critical for maintenance of synaptic transmission and play an important role in the modulation of synaptic efficacy (Keyser & Pellmar 1994). Astrocyte (Kastritsis *et al.* 1992), oligodendrocytes (Salter & Hicks 1994) and microglial cells (Walz *et al.* 1994) are all direct targets for extracellular nucleotides via various P_2 purinoceptors, which mediate a variety of biological processes in glial cells. These include activation of the IP/Ca^{2+} system (Kastritsis *et al.* 1992, Salter & Hicks 1994, Lin & Chuang 1993, Lin & Chuang 1994), increase in prostaglandin synthesis (Gebicke-Haerter *et al.* 1988), thromboxane release (Bruner & Murphy 1993), inhibition of agonist-stimulated cAMP accumulation (Debernardi *et al.* 1993a, Boyer *et al.* 1993), changes in morphology (Neary & Norenberg 1992), activation of ion channels (Walz *et al.* 1993, Walz *et al.*

1994, Nörenberg *et al.* 1994, Langosch *et al.* 1994) and increase in glial fibrillary acidic protein and DNA synthesis (Neary *et al.* 1994, Neary *et al.* 1994, Abbracchio *et al.* 1994). In addition, a 53 kDa protein was identified on astrocyte membranes by immunoblotting with an antibody raised against a putative rat fibroblast P_{2U} receptor (Bruner & Murphy 1993). These data clearly suggest an important role for extracellular nucleotides in the neuron-glia interaction and in the regulation of glial cell functions.

1.5 Summary

A wealth of data - particularly the recent recording of synaptic potentials between neurons, and the cloning of four P_2 purinoceptors - have unambiguously demonstrated that ATP and other nucleotides are ubiquitous extracellular mediators involved in many biological processes. The superfamily of P_2 purinoceptors can be divided into two main groups:- ligand-gated ion channels (P_{2X}) and G protein-coupled receptors (P_{2Y}, P_{2U}, P_{2T} and P_{2D}), with P_{2Z} receptors, forming a separate category of apparent ion channels/membrane pores. Multiple P_2 receptors are expressed in the neurons as well as in a variety of non-neuronal cells. After long controversy, the extracellular nucleotide field is now established, and is rapidly becoming one of the most fascinating areas of transmitter research. The physiological and pathological significance of P_2 receptors still awaits full-scale exploration.

Chapter 2. GENERAL METHODS

A list of materials used in the following methods and their suppliers is provided at the end of this chapter unless otherwise specified.

2.1 Cell Culture

2.1.1 Rat Hypothalamic neurons

Foetal rat hypothalami were harvested and dispersed essentially as described(Hu *et al.* 1992). A Sprague-Dawley rat on days 18-20 of pregnancy (the day of mating referred to as day 1) was decapitated and the abdomen was cleaned with 70% ethanol and cut open. The uterus (usually containing 11 - 15 fetuses) was removed and placed into dissection solution (Table 2-1) in a sterile plastic petri dish. Fetuses were then removed from the uterus and decapitated and fetal heads placed onto a piece of 3M filter paper soaked in dissection solution in a petri dish. One mid cut was made through the dorsal skull and the whole fetal brain gently eased out and inverted so that the hypothalamus was visible. The hypothalamus, bordered by the hypothalamic sulci laterally, the mammillary bodies caudally and the optic chiasm rostrally were taken using fine watchmaker forceps and placed into ice-cold collection buffer (Table 2-1). All tissue dissection and cell dispersion instruments were autoclaved before use.

Washed once in fresh collection buffer, the hypothalami were cut into small pieces with a sterile scalpel and digested by dispase II in 5 ml of enzyme solution (Table 2-1) in a shaking waterbath at 37°C for 30 min. After digestion the tissue was gently passed through 20-gauge needles twice to facilitate disruption of tissue and the supernatant collected into 10 ml of collection buffer. The remaining hypothalamic fragments were digested once more as described above. The collected supernatant was sieved through a mesh with openings of 0.23 mm in diameter and then centrifuged at 1000 rpm for 8 min. The cell pellet was resuspended in 2 ml of collection buffer, and this cell

suspension was transferred into 20 ml of debris removal buffer (Table 2-1) and centrifuged again.

The final cell pellet was resuspended in culture medium (Table 2-1), plated at a density of 2 x $10^5/cm^2$ on 22 mm diameter, poly-L-lysine coated glass coverslips, and maintained at 37°C in a water-saturated atmosphere of 5% CO_2 in air. The culture medium remained unchanged for the first 4 days of culture, after which the serum content of the medium was reduced to 2.5% and half the medium was changed every 3 days. In order to suppress the growth of non-neuronal cells, 10 μM cytosine arabinoside was added to the medium for 30 hours between days 4 and 5. After 5 days in culture, hypothalamic cells formed a neuronal network and after 7 days, elaborate cell connections with long neurites were observed.

2.1.2 Rat Pituitary Cells

To obtain rat pituitary glands, rats were decapitated, head and neck fur trimmed back with scissors and the cranium cut open widely with bone forceps. A steel spatula was inserted under the frontal lobes to rotate the brain posteriorly out of the cranium leaving the pituitary gland exposed under the diaphragm sella. A sterile 19-gauge needle was used to split the diaphragm and score the medial sides of the cavernous sinuses on either side so that the pituitary was impaled, lifted from the pituitary fossa and placed in 5 ml of ice-cold HBSS containing 0.1% BSA, 20 mM HEPES, 100 unit/ml penicillin and 100 μg/ml streptomycin.

Pituitary glands were washed once in HBSS, transferred into a sterile petri dish with a few drop of HBSS and cut into small pieces with a sterile scalpel. The minced pituitary tissue was then transferred to a sterile Bijou bottle, washed in 4 ml of HBSS to remove blood and digested in 3 ml of HBSS containing trypsin (0.125% w/v), DNase I (0.5 mg/ml), BSA (0.1%) and HEPES (20 mM) at 37°C in a shaking waterbath. After 20 min of digestion the tissue fragments were passed through a 10 ml-pipette several times

Table 2-1. Solutions used in culture of rat hypothalamic neurons *

dissection solution	saline		
	50	unit/ml	penicillin
	50	µg/ml	streptomycin
collection buffer	Ca/Mg-free Hanks' balanced salt solution (HBSS)		
	0.1	% w/v	bovine serum albumin (BSA)
	20	mM	HEPES
	5.6	mM	D-glucose
	50	unit/ml	penicillin
	50	µg/ml	streptomycin
enzyme solution	2.4	units/ml	dispase II in phosphate buffered saline
	0.5	mg/ml	DNase I
debris removal buffer	collection buffer		
	4	%	BSA
culture medium	Dulbecco's modified Eagle's medium/nutrient mix F12		
	10	%	fetal calf serum (FCS)
	100	unit/ml	penicillin
	100	µg/ml	streptomycin
	0.25	µg/ml	fungizone

*: Items are ordered as appeared in the text.

and the supernatant was harvested into 20 ml of culture medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS, 5% horse serum, 100 unit/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine). The remaining tissue fragments were subject to further rounds of enzymatic digestion as described above. Usually, it took 4 - 5 runs to complete dispersion. Dispersed cells were plated out on 22 mm diameter glass coverslips in culture medium at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

2.1.3 Gonadotrope-derived Cell Line (αT3-1 Cells)

αT3-1 cells were kindly provided by Dr P. Mellon (Department of Reproductive Medicine, University of California, San Diego, CA, USA), and maintained in DMEM supplemented with 5% FCS, 5% horse serum, 100 unit/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37°C in a water-saturated atmosphere of 5% CO_2 in air and passaged at 7-day intervals by trypsinization.

2.2 Real-time Intracellular Calcium Ion Imaging at the Single Cell Level

The introduction of fluorescent Ca^{2+} indicators such as Fura-2 over the last decade has revolutionized the measurement of intracellular Ca^{2+}. In this study Fura-2 was used to examine intracellular Ca^{2+} signalling at the single cell level in many cell types in a computerized real-time Ca^{2+} imaging system (Figure 2-1).

Fura-2, the most popular fluorescent Ca^{2+} indicator available today, is a dual excitation dye that will fluoresce at two different excitation wavelengths when bound to calcium ions (Figure 2-2). The peak fluorescence occurs with an excitation wavelength of approximately 380 nm and 340 nm for the low and high Ca^{2+} concentrations, respectively. 340 and 380 nm excitation wavelength are used to generate two different fluorescence outputs, which are ratioed so that Ca^{2+} concentrations can be determined

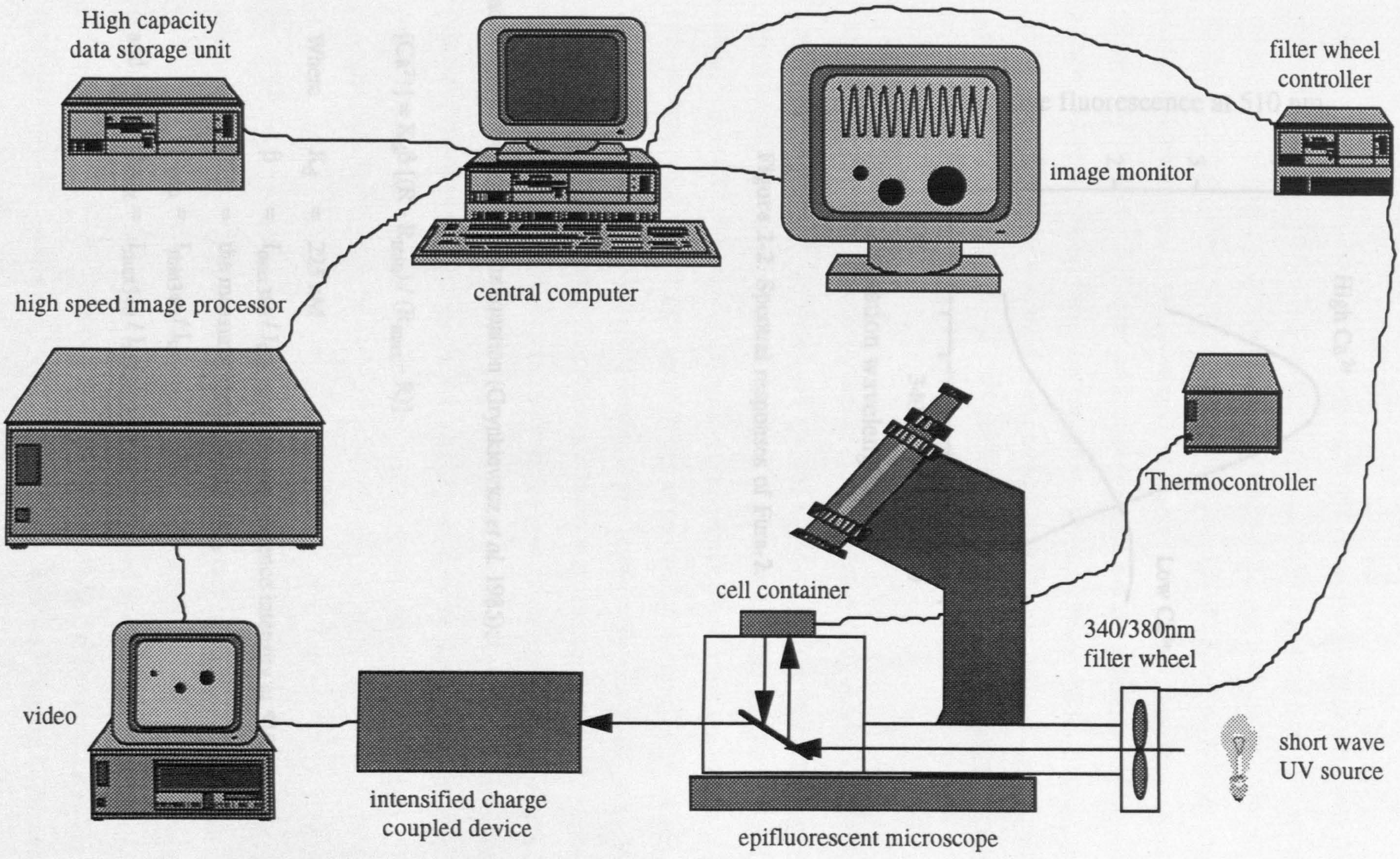


Figure 2-1. Schematic diagram of intracellular calcium ion imaging system

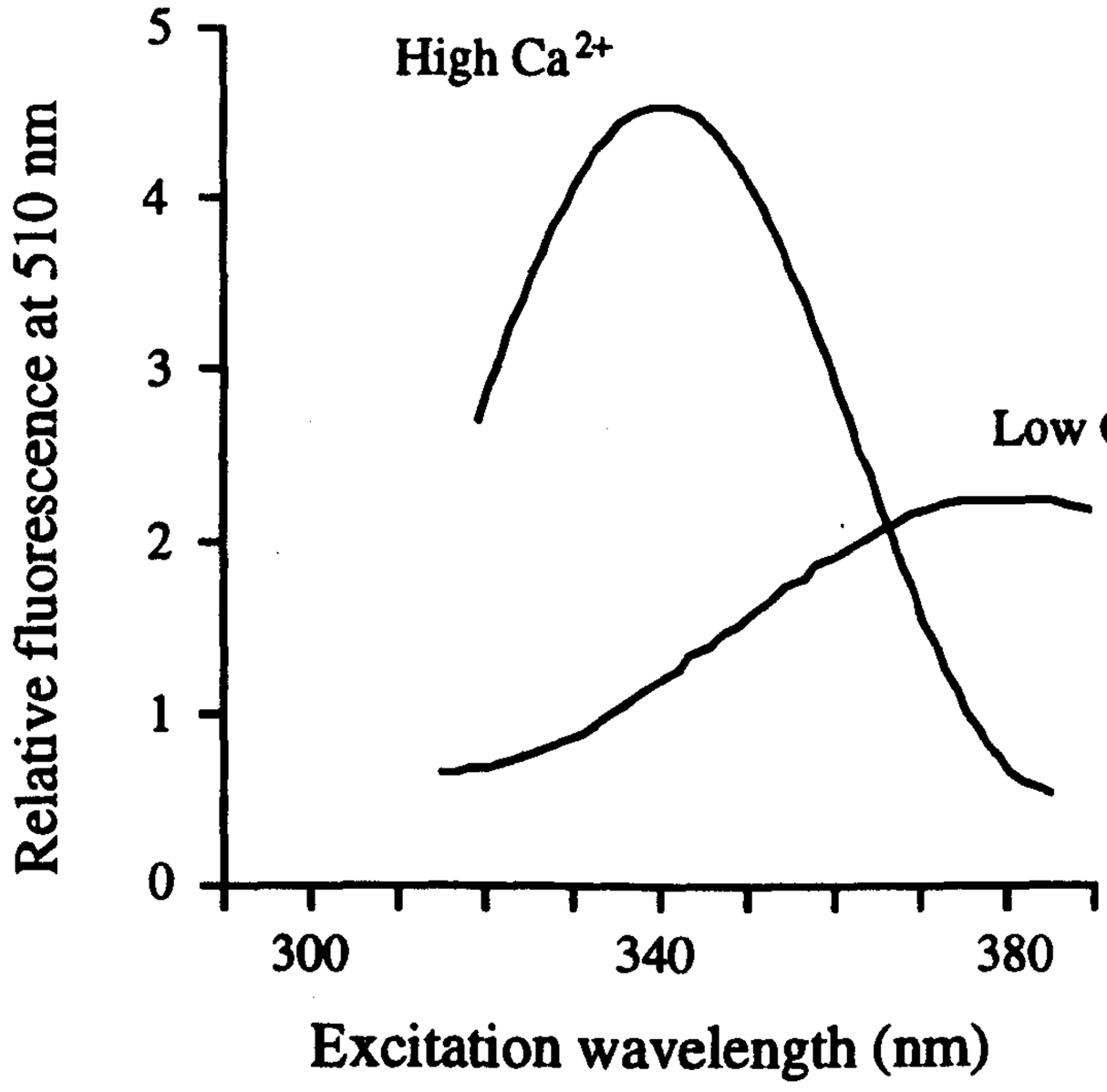


Figure 2-2. Spectral responses of Fura-2.

according to the Grynkiewicz equation (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}] = K_d\beta\,[(R - R_{min}) / (R_{max} - R)]$$

Where K_d = 225 nM

β = I_{max380} / I_{min380} (I = fluorescence intensity at 510 nm)

R = the measured ratio of I_{340} / I_{380}

R_{min} = I_{min340} / I_{max380}

and R_{max} = I_{max340} / I_{min380}.

For Ca^{2+} imaging experiments, cells were cultured on sterile thin glass coverslips (22 mm in diameter, No 1 1/2). Before Ca^{2+} imaging, they were washed with Ca^{2+} buffer containing 130 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES and 0.1% BSA and loaded with Fura-2 AM in the above buffer for about 30 - 75 min (dependent on cell types) at 37°C. Fura-2 AM is the ester form of Fura-2 and can diffuse across the cell membrane into the cytosol where it is rapidly de-esterified by non-specific cytoplasmic esterases resulting in the membrane-impermeable free acid form. After loading, the cell was thoroughly washed with the Ca^{2+} buffer to remove any excess Fura-2 AM and subject to imaging data collection within 30 min.

Real time Ca^{2+} imaging was performed as previously described (Mason *et al.* 1990, Kato *et al.* 1992) at 35°C using λ 340 nm and λ 380 nm excitation alternating at approximately 0.6Hz (i.e. approximately 3.4 seconds between ratioed images), although up to 25Hz was used to estimate response times. The emitted fluorescent at λ 510 nm was passed to an image-intensifying charge-coupled device (ICCD) camera. The resulting fluorescence images at each excitation wavelength were averaged 8 - 16 times in real time, digitized to yield 256 grey levels and captured as 256 x 256 pixel images using the MagiCal system and 'TARDIS' software package supplied by Applied Imaging International Ltd.. On a pixel-by-pixel basis, the ratio of emitted fluorescence (λ340nm/λ380nm) was calculated for each frame after background subtraction, automatically compared with a calibration curve and converted to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

The Ca^{2+} imaging system was calibrated by imaging the cells of interest under artificially raised or reduced intracellular Ca^{2+} concentrations. After loaded with Fura-2 AM, cells were imaged in the elevated extracellular Ca^{2+} concentration of 10 mM and the saturated intracellular Ca^{2+} concentration was achieved by addition of ionomycin (2 μM), which causes a rapid and sustained rise in intracellular Ca^{2+} concentration. The same cells were then washed and bathed in the Ca^{2+}-free medium containing 5 - 20 mM

EGTA, which leads to Ca^{2+} efflux and almost elimination of intracellular free Ca^{2+} within approximately 1 hour. By this mean, the above-described parameters of R_{max}, R_{min} and β were obtained and an individual calibration curve was produced for each type of cell of interest. An example of calibration curve for cultured hypothalamic neurons is illustrated in Figure 2-3.

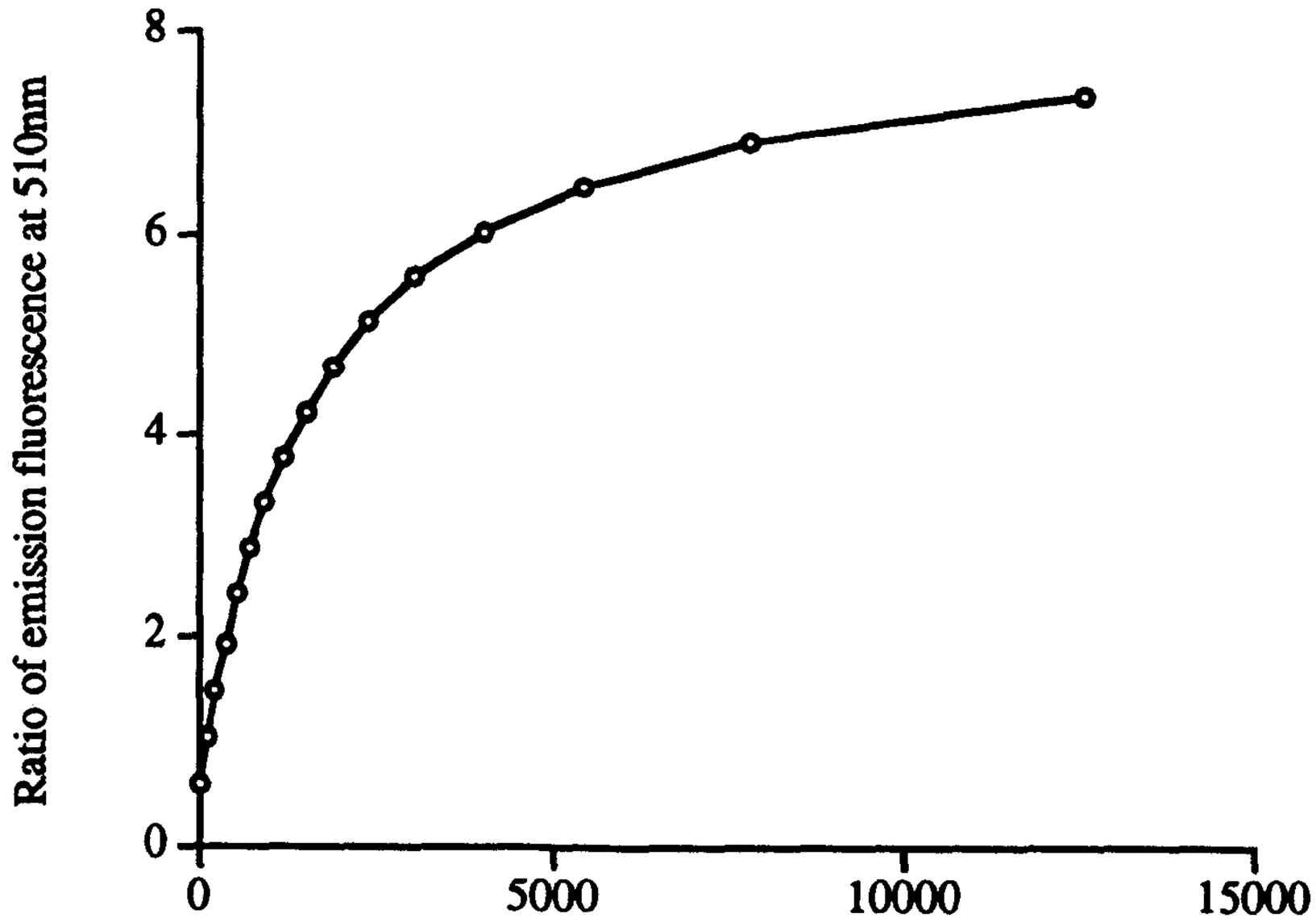


Figure 2-3. Ca^{2+} imaging calibration curve for cultured hypothalamic neurons

2.3 Immunocytochemistry

Immunocytochemistry was performed using the avidin:biotin complex method with peroxidase substrate (ABC-P) essentially as described (Mikkelsen & O'Hare 1991). Experiments were carried out at room temperature unless specified. Cells on glass coverslips were washed in HBSS for 2 x 5 min and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min. After rinsed in 2 ml of KPBS buffer (Table 2-2) for 3 x 10 min, cells were incubated sequentially in 1 ml of 1% H_2O_2 in methanol for 15 min to block endogenous peroxidase and in 0.5 ml of blocking buffer for 20 min. Cells were then incubated with the primary antibody at appropriate dilutions in antibody buffer (Table 2-2) at 4°C for 24 hours. Cells were afterwards rinsed in washing buffer for 3 x 10 min and incubated with appropriate biotinylated secondary antibody (1:400 dilution) in antibody buffer for 1 hour. Cells were rinsed again in washing buffer for 3 x 10 min and incubated in Dako avidin:biotin:peroxidase mixture for 1 hour. The avidin:biotin:peroxidase mixture was then removed by sequentially rinsing in washing buffer, KPBS buffer and 0.05 M Tris-Cl buffer (pH 7.6) each for 10 min. Immunostaining was developed by incubating cells in chromagen solution for 1 min, followed by washing cells in distilled H_2O for 2 x 10 min. Finally, cells on coverslip were mounted onto glass slides with DPX mountant.

For detection of neuron-specific markers microtubule-associated protein 1 (MAP1) in hypothalamic culture, monoclonal anti-rat MAP1 (1:500 dilution), normal rabbit serum and rabbit anti-mouse IgG were used as the primary antibody, normal serum and secondary antibody, respectively, while polyclonal rabbit anti-bovine NSE (1:400 dilution), normal swine serum and swine anti-rabbit IgG used correspondingly for detection of neuron specific enolase (NSE).

Table 2-2. Solutions used in ABC-P immunocytochemistry *

KPBS buffer	PBS		
	2.6	mM	KCl
blocking buffer	KPBS		
	5	%	normal serum
	1	%	BSA
	0.3	%	Triton-X-100
antibody buffer	KPBS		
	1	%	BSA
	0.3	%	Triton-X-100
washing buffer	KPBS		
	0.25	%	BSA
	0.1	%	Triton-X-100
chromagen solution	0.05	M	Tris-Cl buffer (pH 7.6)
	0.04	%	diaminobenzidine tetrahydrochloride
	0.001	%	H_2O_2

*: Items are ordered as appeared in the text.

2.4 Superfusion of Pituitary Cells

Rat pituitary glands were acquired and enzymatically dispersed using a similar procedure to that described above for pituitary cell culture but the enzyme solution for tissue digestion was comprised of 1 mg/ml collagenase, 0.5 mg/ml hyaluronidase, 25 µg/ml DNase, 0.3% BSA in medium M199 (McArdle & Poch 1992). Aliquots of cell suspension containing approximately 3 - 4 x 10^6 cells in culture medium M199 supplemented with 5% FCS, 5% horse serum, 0.3% BSA, 2 mM glutamine, 20 mM HEPES and 50 µg/ml gentamycin were plated out on a 12-well culture plate containing 15 mg Cytodex I beads, which had been pre-swollen in phosphate-buffered saline and autoclaved. Cells were maintained at 37°C in a humidified air atmosphere.

Superfusion experiments were performed as previously described (McArdle & Poch 1992). After 2 days in culture, cells and beads were transferred to superfusion columns (5 x 25 mm Bio-Rad Econocolumns) and superfused with medium M199 containing 0.3% BSA, 1.8 mM Ca^{2+}, 2 mM glutamine and 20 mM HEPES at 37°C at the rate of 0.65 ml/min. A schematic diagram of the superfusion system used is shown in Figure 2-4. A 90 min-superfusion was carried out to wash cells before the start of superfusate collection. Superfusate fractions were collected for periods of 1 - 9 min and stored at -20°C while awaiting determination of pituitary hormone by radioimmunoassay (RIA). The experiments were performed at 37°C. The dead space in the superfusion system was approximately 0.4 ml, which resulted in a time lag of approximately 40 sec between changes of medium and collection of superfusate containing the new medium. This time lag was not corrected for the data presented in this thesis.

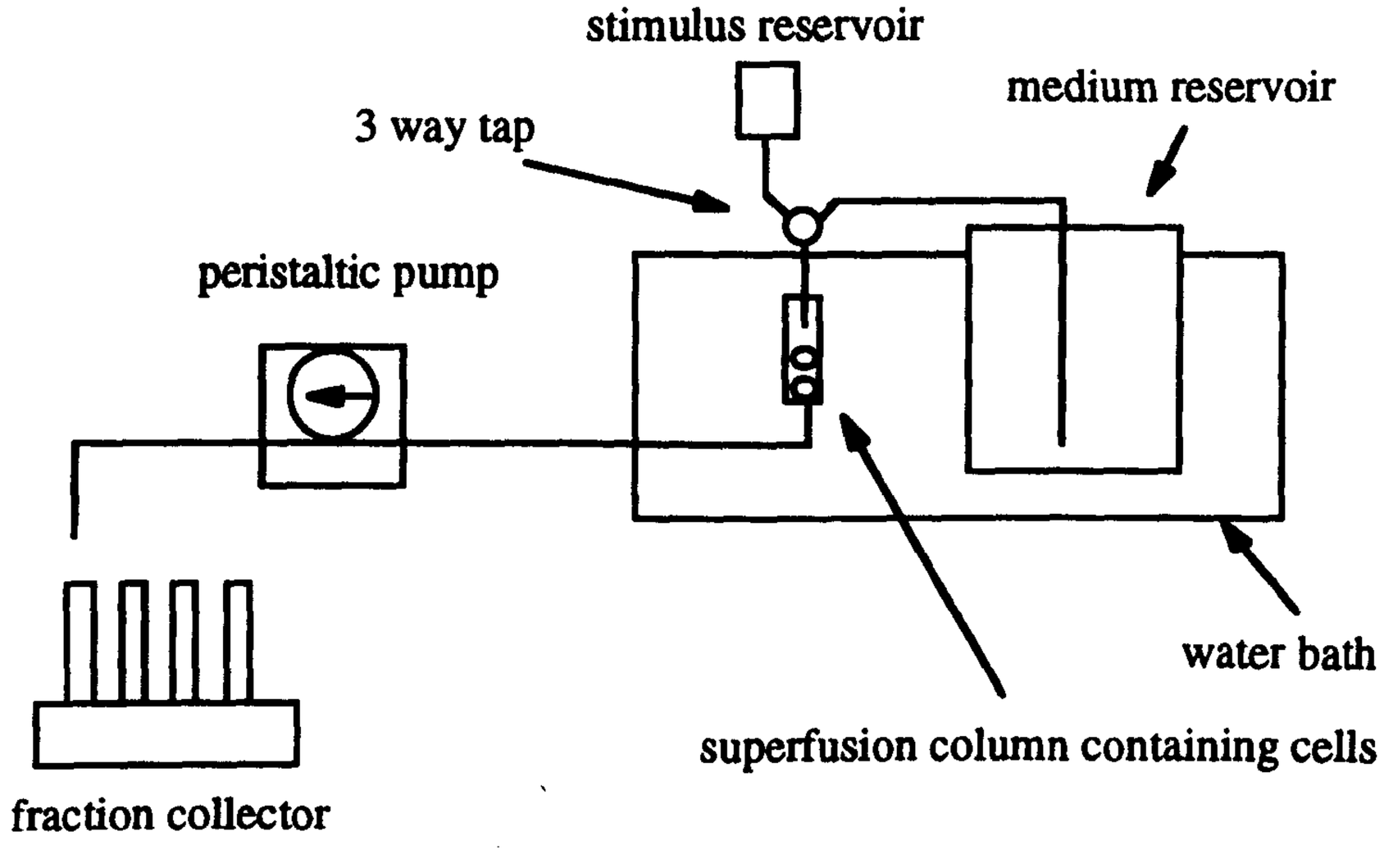


Figure 2-4. Schematic diagram of the superfusion system.

2.5 Luteinizing Hormone (LH) Radioimmunoassay

LH RIA was carried out as described (McArdle & Poch 1992) and is shown in Table 2-3. 200 µl of superfusate collected in the superfusion experiments of rat pituitary cells were used per tube and the assay performed in duplicate. Rat LH standard (NIDDK-rLH-RP3) was diluted 1:1 sequentially in 200 µl of superfusion medium (i.e. medium M199 containing 0.3% BSA, 2 mM glutamine and 20 mM HEPES) to construct standard curve with amounts of LH ranging from 10 pg to 5000 pg per tube. Rabbit anti-rat LH antibody (NIDDK-anti-rLH-S10) in 100 µl of superfusion medium (final titre of 1:18000) was added to each tube except nonspecific binding and total count tubes. After addition of radiolabelled LH ([^{125}I]LH: ~ 20000 cpm in 100 µl of superfusion medium) to each tube, samples were mixed, covered with parafilm and incubated at room temperature for 24 hours. 500 µl of precipitant containing 20% PBS,

Table 2-3. Luteinizing hormone radioimmunoassay

Tube	Standard	Sample	Medium	Anti-LH	[^{125}I]LH	Precipitant
total					100 µl	
NSB			300 µl		··	500 µl
Bo			200	100 µl	··	··
10 pg	200 µl			··	··	··
20 pg	··			··	··	··
40 pg	··			··	··	··
80 pg	··			··	··	··
156 pg	··			··	··	··
312 pg	··			··	··	··
625 pg	··			··	··	··
1250 pg	··			··	··	··
2500 pg	··			··	··	··
5000 pg	··			··	··	··
sample 1		200 µl		··	··	··
sample 2		··			··	··
··						
etc						

7.5% polyethylenglycol-6000, 0.1% Triton-X-100, 0.1% normal rabbit serum and 1% donkey anti-rabbit serum (added immediately before use) were then added to all tubes except total count tubes. Samples were mixed, incubated at room temperature for 15 min and then separated by centrifugation (3000 rpm, 4°C, 20 min). Supernatant was decanted and the pellet counted for 1 min in a gamma counter.

Rat LH standard and rabbit anti-rat LH antibody were kindly supplied by the National Institute of Diabetes, Digestive and Kidney Diseases, USA and [^{125}I]LH prepared by chloramine-T iodination of pure rat LH (NIDDK-rLH-I9) was kindly provided by Miss A. Poch (Institute for Hormone and Fertility Research, Hamburg, Germany).

2.6 Measurement of cAMP Accumulation

Total (intra- and extracellular) cAMP was extracted and measured essentially as described (McArdle *et al.* 1994). Cells cultured on 24-well plates were washed once with a basic salt solution containing in mM: 135 NaCl, 1 KCl, 1.5 $CaCl_2$, 0.5 $MgCl_2$, 1 KH_2PO_4, 5 KOH, 5.6 glucose, 10 HEPES and 0.1% BSA (henceforth referred to as BSS) and preincubated in 0.25 ml of BSS with 0 or 0.25 mM 3-isobutyl-1-methylxanthine (IBMX) at 37°C for 10 min. Cells were then incubated at 37°C in 0.25 ml BSS containing various stimuli as indicated for 10 or 30 min at the presence of 0 or 0.25 mM IBMX. Incubation was terminated by adding 1 ml of ice-cold ethanol and cAMP was extracted by incubating cells at -20°C for 30 min. Cells were then centrifuged at 120 x g for 5 min at room temperature and samples of supernatant of 800 µl were transferred, without disturbing precipitate, into glass tubes. Samples evaporated to dryness at 55°C in vortex under vacuum for 40 min and were resuspended in 0.5 ml of medium M199 containing 10 mM HEPES (referred to as M199-HEPES). Samples containing large amount of cAMP, e.g. in the case of αT3-1 cells stimulated with forskolin and pituitary adenylate cyclase-activating polypeptide 38 (PACAP38), were diluted further before acetylation. 1:50 dilution was given for these samples.

cAMP RIA standards and samples were acetylated by adding 25 µl of acetylation reagent (1 volume anhydrous acetic acid and 2.5 volumes triethylamine) to 500 µl of standards or samples in glass tubes and mixed immediately. cAMP was assayed using a

cAMP RIA kit, kindly provided by IBL, Hamburg, Germany and the procedure was similar to that described above for LH RIA. Acetylated standards were prepared by 1:1 sequential dilution in 100 µl of M199-HEPES to give concentrations of 10 fmol to 1280 fmol per tube. 100 µl of acetylated samples were used per tube and the assay performed in duplicate. 25000 cpm of [^{125}I]cAMP in 100 µl of M199-HEPES and 200 µl of cAMP antiserum in cAMP buffer (Table 2-4) with a final titre in RIA of 1:96000 were used. Samples were incubated at 4°C for 24 hours, terminated by addition of 1 ml of precipitant (supplied by the manufacturer), mixed thoroughly and centrifuged (3000 rpm, 4°C, 20 min). Supernatant was decanted and pellet counted for 1 min in a gamma counter.

Table 2-4. cAMP buffer

Solution 1:	Solution 2:
0.5 g thimerosal	30 ml acetic acid
5.0 g BSA	45 g NaCl
19.8 g Na_2SO_4	
18.6 g EDTA	
make to 400 ml in H_2O	make to 400 ml in H_2O and to pH 4.5 with 10 M NaOH

Mix both solutions at 1:1, adjust pH to 5 and bring volume to 1000 ml

2.7 Measurement of cGMP Accumulation

Total (intra- and extracellular) cGMP was extracted and determined by RIA as described (McArdle *et al.* 1993), which was almost the same as one used for the

cAMP assay described above but acetylation of samples was not required. A cGMP RIA kit, kindly provided by IBL, Hamburg, Germany, was used. 100 µl of samples were used per tube and the assay performed in duplicate. 25000 cpm of [^{125}I]cGMP in 100 µl of M199-HEPES and 200 µl of cGMP antiserum in PBS with a final titre in RIA of 1:160000 were used.

2.8 Western Immunoblotting of Protein Kinase C (PKC)

The method used for protein extraction and Western Immunoblotting was kindly provided by Dr M. Kratzmeier (Institute for Hormone and Fertility Research, Hamburg, Germany). Solutions and buffers were prepared as shown in Table 2-5.

2.8.1 Preparation of Cytosol and Particulate Protein Fractions

Cytosolic and particulate fractions were prepared essentially as described by Kiley, et al (Kiley *et al.* 1991). Cells were grown on 75 cm^2 culture flasks and, one day prior to PKC assay, culture medium was replaced. Concentrated stimuli in a small volume (50 µl) were directly added to cells in culture and incubated under the same culture conditions for 10 min. The cells were then washed with 5 ml of ice-cold buffer A and scraped into 1.5 ml of ice-cold buffer B in a 2 ml eppendorf tube on ice. Cells were homogenized on ice by an ultrasonifying tip (Branson, USA) for ~ 7 sec, and the lysate was centrifuged at 100,000 g for 40 min at 4°C. The supernatant was collected as the crude cytosolic fraction and the crude pellet as particulate fraction.

The crude cytosolic fraction was purified as follows: protein was precipitated by adding trichloroacetic acid (10% final), incubating at room temperature for 15 min and subsequently centrifuging at room temperature for 5 min in a bench-top eppendorf centrifuge. After discard of supernatant, the precipitate was washed twice with 1 ml of ether, vacuum-dried for 30 min to remove residual of ether and dissolved in 200 µl of

buffer D by ultrasonification using an ultrasonifying tip for 5 x 1 sec on ice. The sample was centrifuged again and the supernatant (as the cytosol fraction) stored at -20°C until use. Protein concentrations of samples were measured by a BioRad kit according to the manufacturer's instruction.

The crude particulate fraction was resolubilized in 200 µl of ice-cold buffer C by ultrasonification using an ultrasonifying tip for 5 x 1 sec on ice, which was then centrifuged in an eppendorf centrifuge (14000 rpm, 4°C and 30 min). The supernatant (as the particular fraction) was collected and stored at -20°C until use.

Table 2-5. Solutions used for Western immunoblotting *

buffer A	25	mM	Tris-Cl (pH 7.4)
	0.25	M	sucrose
	2.5	mM	Mg-acetate
	1	mM	DL-dithiothreitol (DTT)
buffer B	buffer A		
	2.5	mM	EGTA
	20	µM	leupeptin
	0.1	mM	phenylmethylsulphonyl fluoride
buffer C	buffer B		
	0.5	%	Triton-X 100

(continued)

Table 2-5. Solutions used for Western immunoblotting * (continued)

buffer D	50	mM	Tris-Cl (pH 6.8)
	12	%	glycerol
	1	%	SDS
gel solution	3	M	Tris-Cl (pH 8.4)
	0.3	%	SDS
acrylamide solution	48	%	acrylamide
	1.5	%	bis acrylamide
gel sample buffer	50	mM	Tris-Cl (pH 6.8)
	4	%	SDS
	12	%	glycerin
	0.01	%	Serva Blue G
	2	%	2-mercaptoethanol
anode buffer	0.2	M	Tris-Cl (pH 8.9)
	0.005	%	thimerosol
cathode buffer	0.1	M	Tris-Cl (pH 8.2)
	0.1	M	tricine
	0.1	%	SDS
	0.005	%	thimerosol

(continued)

Table 2-5. Solutions used for Western immunoblotting * (continued)

blotting buffer	0.1	M	Tris-Cl (pH 8.2)
	0.193	M	glycine
	0.005	%	thimerosol
TBS (Tris-buffered saline)	20	mM	Tris-Cl (pH 7.6)
	0.137	M	NaCl
blocking solution	1	%	Boehringer Block Reagent
	0.1	M	maleic acid (pH 7.5)
	0.15	M	NaCl
	0.005	%	thimerosol
TBST (TBS-Tween)	TBS		
	0.05	%	Tween 20
antibody solution	9	volume	TBST
	1	volume	blocking solution

*: Items are ordered as appeared in the text.

2.8.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

For protein electrophoresis, a 10% polyacrylamide separating gel and 4% stacking gel was prepared as shown in Table 2-6 (Schägger & von Jagow 1987).

Samples of cytosolic and particulate fractions with equal amount of protein (40 μg per lane) in gel sample buffer were heat-treated (95°C, 5 min) and, after cooling, loaded into gel. Electrophoresis was run in anode buffer and cathode buffer under 50 V. The Amersham Rainbow™ coloured protein molecular weight markers of 14.3 - 200 kDa were used. The samples were then electrically transferred onto a polyvinylidene

Table 2-6. SDS-polyacrylamide gel for PKC assay

1. Separating gel (cast first):	
glycerol	4 g
H_2O	11 ml
gel solution	10 ml
acrylamide solution	5 ml
Ammonium persulfate (APS: 10% in stock)	150 μl
N,N,N',N'-tetramethylethylenediamine (TEMED)	20 μl
2. Stacking gel (cast over the polymerized separating gel):	
H_2O	8.4 ml
gel solution	3.1 ml
acrylamide solution	1.0 ml
APS (10%)	150 μl
TEMED	20 μl

difluoride membrane (Millipore Immobilon P) in blotting buffer under 1 Ampere for 90 min (Towbin *et al.* 1979). Following electrotransfer the membrane was stained with

0.4% Ponceau S to monitor the transfer efficiency and homogeneity of protein loading and, afterwards, destained in TBS buffer at room temperature for 5 min.

2.8.3 Immunodetection of PKC by Enhanced Chemiluminescence (ECL)

PKC was immunochemically detected using an Amersham ECL kit and GibcoBRL subtype-specific polyclonal rabbit anti-PKCα, PKCζ and PKCε. Experiments were performed at room temperature. After nonspecific blocking treatment in blocking solution overnight at 4°C, membranes were washed in TBST buffer for 10 min and incubated with the primary antibodies at 1:200, 1:300 and 1:1000 dilutions for rabbit anti-PKCα, PKCζ and PKCε, respectively, in antibody solution for 1 hour. Membranes were then washed in TBST for 3 x 10 min and exposed to peroxidase-conjugated goat-anti-rabbit IgG antibody (secondary antibody, 1:1000 dilution) in antibody solution for 1 hour. Membranes were washed again in TBST for 3 x 10 min and rinsed in H_2O. The PKC bands were visualized by immerging the membrane into 20 ml of the Amersham ECL detection reagent for 1 min and immediately exposing to Fuji RX 400 films for 30 seconds. For quantification, the intensity of bands on film was analysed using a video imaging-based densitometric system (Jandel Scientific Software Ltd, Germany).

2.9 Molecular Cloning

2.9.1 mRNA Extraction

Total RNA from rat pituitary glands was isolated using the RNAzol™ B solution containing guanidinium thiocyanate and phenol. Pituitary glands were homogenized in RNAzol™ B (2 ml per 100 mg tissue) with a few strokes in a glass-Teflon homogenizer. 0.1 ml of chloroform were added to each 1 ml of homogenate and mixed by shaking vigorously for 15 sec. After staying on ice for 15 min, the sample was

centrifuged (12,000 g, 4°C, 15 min) and the upper aqueous phase transferred to a fresh tube, to which an equal volume of isopropanol was added. The sample was then incubated on ice for 15 min and centrifuged again. The supernatant was discarded and the RNA pellet washed with 1 ml of 70% ethanol by vortexing and subsequent centrifugation (75,000 g, 4°C, 8 min). The pellet of total RNA dried under vacuum for 10 min and was dissolved in diethylpyrocarbonate (DEPC)-treated RNase free H_2O. mRNA was then extracted by passing the sample of total RNA through a Stratagene Poly(A) Quik mRNA Isolation column of oligo(dT) cellulose and dissolved in H_2O and stored at -80°C. When working with RNA, a great deal of care was taken to avoid contamination by RNase and DEPC-treated solution used where appropriate.

2.9.2 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

mRNA was used as templates to synthesize the single strand cDNA by reverse transcriptase and such cDNA then served as templates to amplify a gene of interest by PCR technology. The Pharmacia first-strand cDNA synthesis system was used. 100 ng of rat pituitary mRNA in 20 µl H_2O was heated to 65°C for 10 min and then chilled on ice. To a fresh RNase free eppendorf tube the followings were added in order and mixed:

Bulk 1st-strand reaction mix	11 µl
Random hexadeoxynucleotides ($pd(N)_6$) primer (0.2 µg/µl)	1 µl
DTT (200 mM)	1 µl
Heat-denatured mRNA	20 µl

Reverse transcription was performed by incubating the above mixture at 37°C for 1 hour. The bulk 1st-strand reaction mix was supplied by the manufacturer and contained reverse transcriptase, BSA, dATP, dCTP, dGTP, and dTTP.

PCR was then performed as follows:

1. 10 µl of the completed 1st-strand cDNA reaction product in a 0.5 ml PCR tube was heated to 90°C for 5 min and then chilled on ice.
2. 5 µl of 10 x PCR buffer (100 mM Tris-Cl (pH 8.3), 500 mM KCl, 25 mM $MgCl_2$, 0.01% gelatin), 1 µl of each PCR primers (see below) and 32.8 µl H_2O were added.
3. The sample was heated to 80°C for 2 min.
4. 0.2 µl of AmpliTaq DNA polymerase (5 units/µl) was then added, followed by a drop of mineral oil.
5. PCR was set for 25 thermal cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2.5 min in a DNA Thermal Cycler.
6. After completion, the PCR product was analysed in a 1.2% agarose gel in 0.5 x TBE buffer (Table 2-7).

In this study two degenerate PCR primers (295 ng/µl in H_2O) used were

5'-CTCACC(/G)TGCATA(/C)AGCGTGCA-3' and

5'-C(/G)TC(/G)TG(/C)CCCT(/G)GCCAGGAAGTA-3'.

2.9.3 Cloning

After PCR amplification, the cDNA of interest was cloned into the vector pCR™II using Invitrogen's TA Cloning System (version 1.3). Ligation was performed as follows: in a tube containing 1 µl of 10x ligation buffer (Table 2-7) and 5 µl of H_2O, 1 µl of PCR product, 2 µl of vector pCR™II (25 ng/µl) and 1 µl of T4 DNA ligase (4 units/µl) were added and the reaction carried out at 12°C overnight. 1 µl of the completed ligation product was added to a tube containing 2 µl of 0.5 M β-mercaptoethanol and 50 µl of competent *E. coli* INVαF' cells and incubated on ice for 30 min. The sample was heat-shocked at 42°C for exactly 45 sec, followed by incubation on ice for further 2 min. After addition of 450 µl of pre-warmed SOC medium (Table 2-7), the sample was incubated at 37°C for 1 hour at 225 rpm in a

shaker-incubator. 25 µl and 100 µl of the sample were spread on separate LB agar plates containing ampicillin (50 µg/ml) and 5-bromo-4-chloro-3-indolyl-D-galactoside (X-Gal: 1 mg/plate) and the plates were incubated at 37°C overnight. White colonies were picked and grown up in LB medium (Table 2-7) containing ampicillin (50 µg/ml) at 37°C overnight at 225 rpm in a shaker-incubator for plasmid isolation.

A small scale plasmid DNA preparation was performed according to the method described by Sambrook, et al (Sambrook *et al.* 1989). 1.5 ml of overnight bacterial culture were centrifuged (12000 g, 4°C, 30 sec) and the bacterial pellet was resuspended in 100 µl of solution I (Table 2-7) and alkalytically lysed by adding 200 µl of solution II (Table 2-7) and incubating on ice for 3 min. The sample was neutralized by adding 150 µl of solution III (Table 2-7) and by subsequent centrifugation (12,000 g, 4°C, 5 min). The supernatant was further deproteinized by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuging at 4°C for 2 min at 12000 g. Supernatant was collected and its DNA was precipitated by adding two volume of ethanol and incubating at room temperature for 2 min. After centrifugation (12,000 g, 4°C, 5 min) and removal of supernatant, the DNA pellet was washed with 70% ethanol, dried under vacuum for 10 min and dissolved in 20 µl of TE buffer (Table 2-7). DNA concentrations were measured in a mini-DNA/RNA spectrometer.

The obtained plasmid DNA was then subject to restriction enzyme analysis. In this study, restriction enzyme EcoRI was used to cut both ends of the insert in the pCR™II vector. 1 µg of the plasmid DNA was cut by 10 units of EcoRI in a total volume of 20 µl in the Gibco REact® 3 buffer (Table 2-7) at 37°C for 1 hour. After heat-inactivation (75°C, 10 min), the sample was run in a 1% agarose gel in 0.5x TBE buffer.

After confirmation of the insert in pCR™II vector, a midi-scale of plasmid DNA preparation was carried out using the QIAGEN Plasmid Midi Kit to yield ~ 100 µg of pure plasmid DNA for use in further experiments. 50 ml of overnight bacterial culture were centrifuged (6,000 g, 4°C, 10 min) and the pellet was resuspended in 4 ml of

buffer P1 (Table 2-7), alkalytically lysed by adding 4 ml of buffer P2 (Table 2-7) and incubating at room temperature for 5 min. The sample was neutralized by adding 4 ml of chilled buffer P3 (Table 2-7) and incubating on ice for 15 min, followed by centrifuging at 4°C for 30 min at 30,000 g. The supernatant was promptly collected and passed through a QIAGEN-tip 100 by gravity, which had been equilibrated with 4 ml of buffer QBT (Table 2-7). The tip was washed with 2 x 10 ml of buffer QC (Table 2-7) and the plasmid DNA eluted with 5 ml of buffer QF (Table 2-7). The DNA in buffer QF was then precipitated by adding 0.7 volumes of isopropanol and centrifuging at 4°C for 30 min at 15,000 g. The DNA pellet was washed with 5 ml of 70% ethanol, dried under vacuum for 10 min and dissolved in 100 µl of TE buffer.

2.9.4 DNA Sequencing

The insert in the pCR™II vector was sequenced by chain-termination method using the USB Sequenase Version 2.0 DNA Sequencing Kit. Plasmid DNA (4 µg) was denatured by adding 0.1 volumes of 2 M NaOH, 2 mM EDTA and incubating 30 min at 37°C (Lim & Pene 1988). The mixture was neutralized by adding 0.1 volumes of 3M Na-acetate (pH 5.2) and the DNA precipitated with 3 volumes of ethanol (-70°C, 15 min). The pelleted DNA was washed with 70% ethanol, vacuum-dried and dissolved in 7 µl H_2O, to which 2 µl of Sequenase Reaction Buffer and 1 µl of sequencing primer were added. This DNA mixture was heated to 65°C for 2 min and then cooled slowly to < 35°C over 20 min. The labelling reaction was done by adding 1 µl of 0.1 M DTT, 2 µl of labeling mix of dGTP, dCTP and dTTP (all 1.5 µM), 0.5 µl of [^{35}S]dATP (10 µCi/µl or 1000 Ci/mmol), and 2 µl of Sequenase Polymerase (1.6 units/µl) to the DNA mixture. After incubation at room temperature for 5 min, the labelling reaction was terminated by transferring 3.5 µl of labelling reaction to each termination tube containing 2.5 µl of either of ddATP, ddGTP, ddCTP and ddTTP (all 8 µM) and incubating at 37°C for 5 min. The termination reaction was stopped by adding 4 µl of stop buffer (Table 2-7). The completed samples were heated to 94°C for 1 min immediately before loading onto 6% polyacrylamide sequencing gel (Table 2-7) at 2 µl

per lane. The gel was run in 1x TBE buffer under constant power of 45 W. After electrophoresis the gel was dried at 80°C under vacuum for 2 hours and exposed to Hyperfilm-MP films at room temperature for ~ 2 days.

Table 2-7. Solutions, medium and gel used for molecular cloning *

TBE buffer	89	mM	Tris-base
	89	mM	boric acid
	2	mM	EDTA
10x ligation buffer	60	mM	Tris-Cl (pH 7.5)
	60	mM	$MgCl_2$
	50	mM	NaCl
	0.1	%	BSA
	70	mM	β-mercaptoethanol
	1	mM	ATP
	20	mM	DTT
	10	mM	spermidine
SOC medium	2	%	tryptone
	0.5	%	yeast extract
	10	mM	NaCl
	2.5	mM	KCl
	10	mM	$MgCl_2$
	10	mM	$MgSO_4$
	20	mM	D-glucose

(continued)

Table 2-7. Solutions, medium and gel used for molecular cloning * (continued)

LB medium	1	%	tryptone
	0.5	%	yeast extract
	0.5	%	NaCl
	1	mM	NaOH
solution I	25	mM	Tris-Cl (pH 8)
	50	mM	glucose
	10	mM	EDTA
solution II	0.2	M	NaOH
	1	%	SDS
solution III	3	M	K-acetate
	11.5	% v/v	glacial acetic acid
TE buffer	10	mM	Tris-Cl (pH 8)
	1	mM	EDTA
REact 3 buffer	50	mM	Tris-Cl (pH 8)
	10	mM	$MgCl_2$
	100	mM	NaCl
buffer P1	50	mM	Tris-Cl (pH 8)
	10	mM	EDTA
	100	µg/ml	RNase A
buffer P2	0.2	M	NaOH
	1	%	SDS

(continued)

Table 2-7. Solutions, medium and gel used for molecular cloning * (continued)

buffer P3	3	M	K-acetate (pH 5.5)
buffer QBT	750	mM	NaCl
	50	mM	MOPS (pH 7)
	15	%	ethanol
	0.15	%	Triton X-100
buffer QC	1	M	NaCl
	50	mM	MOPS (pH 7)
	15	%	ethanol
buffer QF	1.25	M	NaCl
	50	mM	Tris-Cl (pH 8.5)
	15	%	ethanol
stop buffer	95	%	formamide
	20	mM	EDTA
	0.05	%	bromophenol Blue
	0.05	%	xylene cyanol FF
50 ml DNA sequencing gel	10	ml	5x TBE buffer
	6	ml	50% long ranger gel solution
	21	g	urea
	x	ml	H_2O (bringing to 50 ml)
	500	µl	10% APS
	12.5	µl	TEMED

*: Items are ordered as appeared in the text.

2.10 Real-time Dynamic Bioluminescence Measurements of ATP Release

Pituitary glands were enzymatically dispersed in Ca^{2+}-free HBSS containing 0.3% BSA, 1 mg/ml collagenase, 25 μg/ml DNase and 10 mM HEPES, as described above. Approximately 2 x 10^5 cells were plated out in a plastic vial with a 2 cm^2 surface area and cultured under medium M199 supplemented with 5% FBS, 5% horse serum, 100 unit/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37°C in a water-saturated atmosphere of 5% CO_2 in air. Real-time dynamic bioluminescence measurements of ATP release on attached cells was performed on the basis of luciferase-catalyzed oxidation of D-luciferin (ATP + luciferin + O_2 $\xrightarrow{\text{luciferase}}$ oxyluciferin + AMP + PP_i + CO_2 + light) (Strehler & McElroy 1957). The method used here was modified from that described by White (White 1977) using a 1250 Luminometer (Bio-Orbit Oy, Turku, Finland). After 2 days in culture, cells were thoroughly washed in aqueous 137 mM NaCl containing 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgSO_4$, 0.4 mM KH_2PO_4, 3 mM Na_2HPO_4, 4 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES (pH 7.4) and 1% BSA (henceforth referred to as ATP assay buffer). Then cells were bathed in 190 μl ATP assay buffer containing highly purified firefly luciferase and synthetic D-luciferin at the final concentrations of 75 and 150 μg/ml, respectively, for 20 min before luminescence measurements at room temperature. Concentrated stimulators or reagents in a volume of 10 μl were very gently added to cells through a 200 μl pipette tip. No luminescence measurement was made during the addition as indicated in illustrations. Emitted light was integrated for 1 second periods and continuously recorded.

2.11 Animals

Sprague-Dawley rats on days 18-20 of pregnancy were provided by the Department of Comparative Biology, Charing Cross Hospital, London and the Medical School, University of Bristol, Bristol. Wistar rats were provided by the Medical School,

University of Bristol, Bristol and the Institute for Hormone and Fertility Research, Hamburg, Germany.

2.12 Materials

Specific materials including instruments were listed in Table 2-8 and other unlisted reagents used in this study were purchased from Sigma, unless otherwise specified.

Table 2-8. Specific materials used

Materials	Suppliers*
Cell culture	
collagenase	Serva
dispase II	Boehringer
DMEM, DMEM/F12, M199, HBSS, FCS, horse serum, fungizone	Gibco
Calcium ion imaging	
Fura-2 AM	Cambridge Bioscience
glass coverslips	Raymond A Lamb
MagiCal system, 'TARDIS' software	Applied Imaging
Immunocytochemistry	
antibodies of anti-MAP1 and anti-NSE	Cambridge Bioscience
DPX mountant	Raymond A Lamb

(continued)

Table 2-8. Specific materials used (continued)

Materials	Suppliers*
normal rabbit serum, normal swine serum,	
biotinylated rabbit anti-mouse IgG,	
swine anti-rabbit IgG	Dako
Superfusion of Cells	
fraction collector	Isco
LH RIA	
LH standard, anti-LH antibody, rat LH (for iodination)	NIDDK
normal rabbit serum, 1% donkey anti-rabbit serum	Scottish Antibody
cAMP and cGMP assay	
cAMP, cGMP RIA kits	IBL
Western Immunoblotting of PKC	
Boehringer Block Reagent	Boehringer
Ponceau S, peroxidase-conjugated goat-anti-rabbit IgG	Sigma
protein electrotransfer	Hoefer
protein gel facilities	Pharmacia
Rainbow™ coloured protein molecular weight markers,	
a polyvinylidene difluoride membrane, ECL kit	Amersham
Serva Blue G	Serva
specific polyclonal rabbit anti-PKCα, PKCζ and PKCε	Gibco

(continued)

Table 2-8. Specific materials used (continued)

Materials	Suppliers*
Molecular Cloning	
1st-strand cDNA synthesis kit, DNA/RNA spectrometer	Pharmacia
DNA sequencing gel facility	International Biotech
EcoRI, REact 3 buffer	Gibco
gel dryer	Bio-Rad
long ranger gel stock solution	AT Biochem
QIAGEN Plasmid Midi Kit	QIAGEN
Poly(A) Quik mRNA Isolation column	Stratagene
RNAzol™ B	Biotecx
[^{35}S]dATP	Du Pont
TA Cloning System	Invitrogen
Taq DNA polymerase, mineral oil, DNA Thermal Cycler	Perkin-Elmer
USB Sequenase Version 2.0 DNA Sequencing Kit,	
Hyperfilm-MP film	Amersham
Other Chemicals	
A23187	Calbiochem
C-type natriuretic peptide (CNP)	Bissendorf
ethanol, methanol, ether	Merck
2-methylthioATP, β,γ-methylene ATP	RBI
phenol:chloroform:isoamyl alcohol (25:24:1)	Sigma
pituitary adenylate cyclase-activating polypeptide 38	
(PACAP38)	Saxon
ryanodine	Calbiochem
suramin	Bayer
U73122	RBI

* Suppliers:

Amersham International plc., Buckinghamshire, UK

Applied Imaging International Ltd., Sunderland, UK

AT Biochem, Malvern, PA, USA

Bayer plc, Berkshire, UK

Bio-Rad Laboratories Ltd., Hertfordshire, UK

Biotecx Laboratories, Inc., Houston, Texas, USA

Bissendorf Biochemicals, Hannover, Germany

Boehringer-Mannheim UK Ltd., East Sussex, UK

Calbiochem (Calbiochem-Novabiochem) (UK) Ltd., Beeston, Nottingham, UK

Cambridge Bioscience, Cambridge, UK

Dako Ltd., High Wycombe, Bucks, UK

Du Pont (UK) Ltd.,Stevenage, Hertfordshire, UK

Gibco BRL Life Technologies, Paisley, UK

Hoefer Scientific Instruments, San Francisco, USA

IBL, Hamburg, Germany

International Biotech Inc., New Heaven, CT, USA

Invitrogen Corp., San Diego, CA, USA

Isco Inc., Lincoln, NE, USA

Merck Ltd., Lutterworth, Leics, UK

NIDDK, National Institute of Diabetes, Digestive and Kidney Diseases, MD, USA

Perkin-Elmer Corp., Norwalk, CT, USA

Pharmacia Biotech, St. Albans, Herts, UK

QIAGEN Ltd., Surrey, UK

Raymond A Lamb, London

RBI (Research Biochemicals International), Natick, MA, USA

Saxon Biochemicals GmbH, Hannover, Germany

Serva Biochemicals, Heidelberg, Germany

Scottish Antibody Production Unit, Carluke, Lanarkshire, UK

Sigma Chemical Company Ltd., Poole, Dorset, UK

Stratagene Ltd., Cambridge, UK

Chapter 3. ATP RECEPTORS IN RAT HYPOTHALAMIC NEURONS

Introduction

Accumulated evidence has suggested that ATP may act as a transmitter or co-transmitter in both peripheral and central nervous system (Burnstock 1972, Burnstock 1990, Edwards *et al.* 1992). ATP has been shown to activate currents in a variety of cell preparations including cardiovascular muscle (Benham & Tsien 1987, Friel & Bean 1988), visceral muscle (Friel 1988), skeletal muscle (Hume & Honig 1986), sensory neurons (Jahr & Jessell 1983, Krishtal *et al.* 1983, Krishtal *et al.* 1988, Bean 1990), and neurons derived from the locus coeruleus (Tschöpl *et al.* 1992) and the nucleus tractus solitarius (Ueno *et al.* 1992a). ATP mimics the effect of nerve stimulation on many smooth muscles (Burnstock *et al.* 1970, Hoyle *et al.* 1990, von Kügelgen *et al.* 1990) and excites rat medulla oblongata vasomotor neurons *in vivo* (Sun *et al.* 1992). ATP-mediated fast excitatory synaptic transmission has also recently been recorded in guinea-pig coeliac neurons and in rat medial habenula neurons (Evans *et al.* 1992, Silinsky *et al.* 1992, Edwards *et al.* 1992).

ATP induces inositol phosphate accumulation and intracellular calcium mobilization in many non-neuronal cell types and neuroblastoma cell lines (Ehrlich *et al.* 1988, Neary *et al.* 1991, Christie *et al.* 1992, Gerwins & Fredholm 1992, Kastritsis *et al.* 1992, Iredale *et al.* 1992a) but little is known about ATP receptor-mediated intracellular signaling in neurons of the central nervous system. The present study was to examine whether ATP could be a potential and genuine transmitter in hypothalamic neuron transmission and if so, how intracellular Ca^{2+} signalling was conducted.

Methods

After a total of 8 to 15 days in culture, rat hypothalamic neurons were subject to

calcium ion imaging at the single cell level. Ligands were dissolved in water or ethanol and DMSO, and diluted in an appropriate buffer before use, giving final solvent concentrations of <0.05% and <0.01% respectively, at which level they were shown to have no detectable effects on $[Ca^{2+}]_i$ when used alone. As data collection time was limited by image processor memory, ligands were added to cells in a heated chamber containing 600 or 800 µl solution by bolus addition of 150 or 200 µl of 5x concentrated solution through a 200µL pipette tip at a fixed position approximately 6 mm away from the studied cell, rather than by superfusion. Final concentrations of ligands in the solution, rather than 5 x stock ligand concentrations are referred to in the text. Ca^{2+}-free solution was made by omitting $CaCl_2$ and adding 0.1 mM EGTA to Ca^{2+} buffer. The osmolarity of all solutions were adjusted to 290 to 300 mOsm and pH 7.4. All cells were shown to respond to other neurotransmitters such as NMDA and kainic acid, and to K^+ (56 mM) at the end of each experiment to confirm viability. Before and between additions, cells were superfused for ≥ 20 min at 1 ml/min with pre-warmed buffer.

To confirm that cells examined were of neuronal origin, the presence of the neuron-specific markers microtubule-associated protein 1 and neuron specific enolase were determined immunocytochemically and shown in Figure 3-1.

Results

ATP-induced intracellular Ca^{2+} response

Addition of 100 µM ATP produced a rapid and transient increase in $[Ca^{2+}]_i$ in a subpopulation (42%) of total 732 cultured rat hypothalamic neurons examined (Figure 3-2a). An approximately 8-fold increase in $[Ca^{2+}]_i$ from a resting level of 163 ± 9.4 nM to a maximum of 1231 ± 88 nM (Means ± SE, n = 96 cells with the initial exposure only to 100 µM ATP) was observed (Figure 3-2b). $[Ca^{2+}]_i$ peaked around 12 s (12 ± 1, n = 96) after ATP addition, falling to half maximum after 35 ± 4 s (n = 96) and to

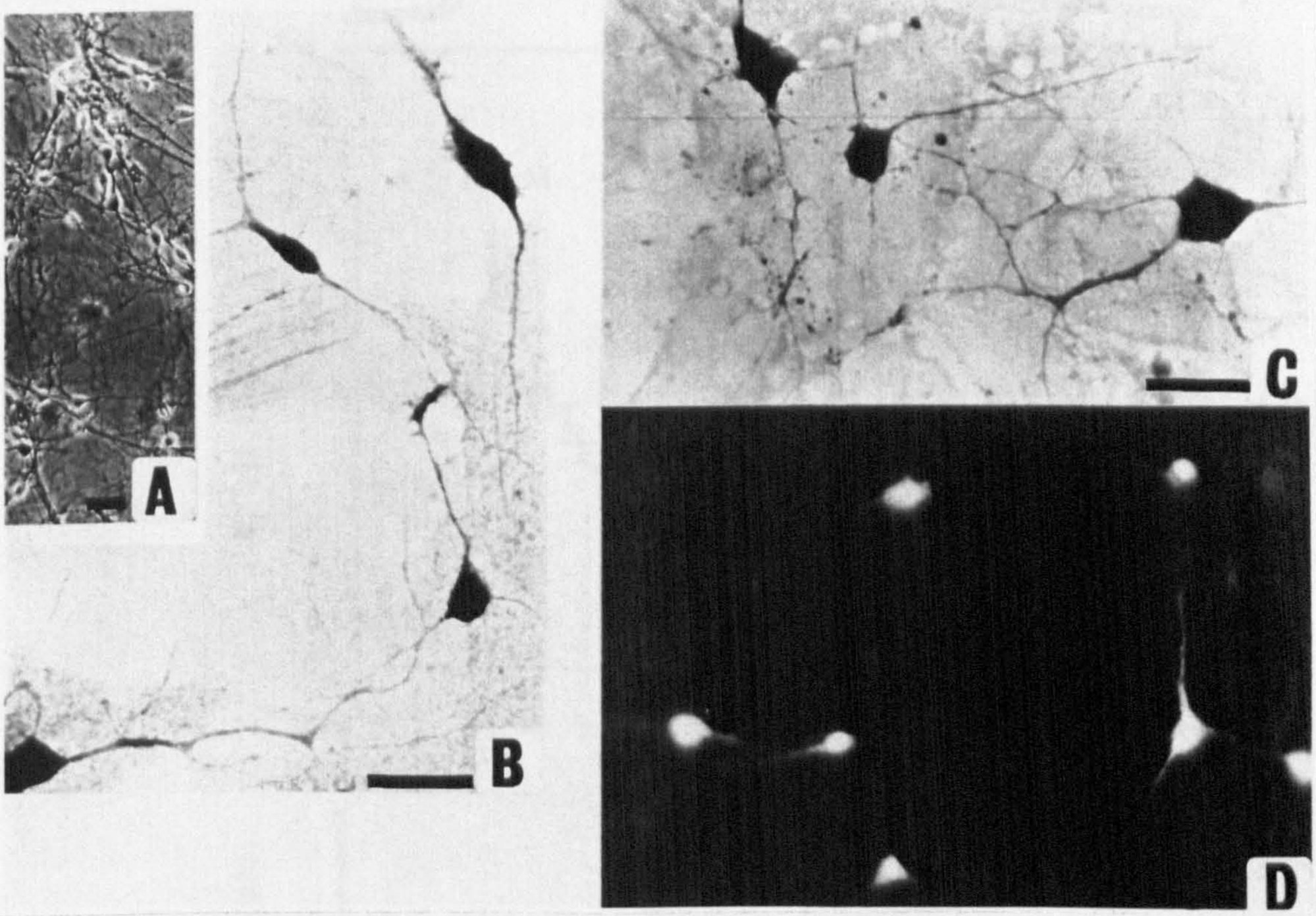


Figure 3-1. Hypothalamic neurons and their neural network formation in primary rat foetal hypothalamic neuronal cultures maintained 10 days. (A) Hypothalamic neurons viewed through phase contrast optics. (B) and (C) Immunocytochemical localization of neuronal specific markers, microtubule-associated proteins 1 (MAP1, 1:500 dilution) and neuron-specific enolase (NSE, 1:400 dilution), respectively. Primary antibodies were replaced with normal rabbit serum (1:100 dilution) for controls (not shown). (D) Hypothalamic neurons loaded with Fura-2 and excited by 340 nm UV light viewed through epifluorescence optics. Scale bars = 40 μM.

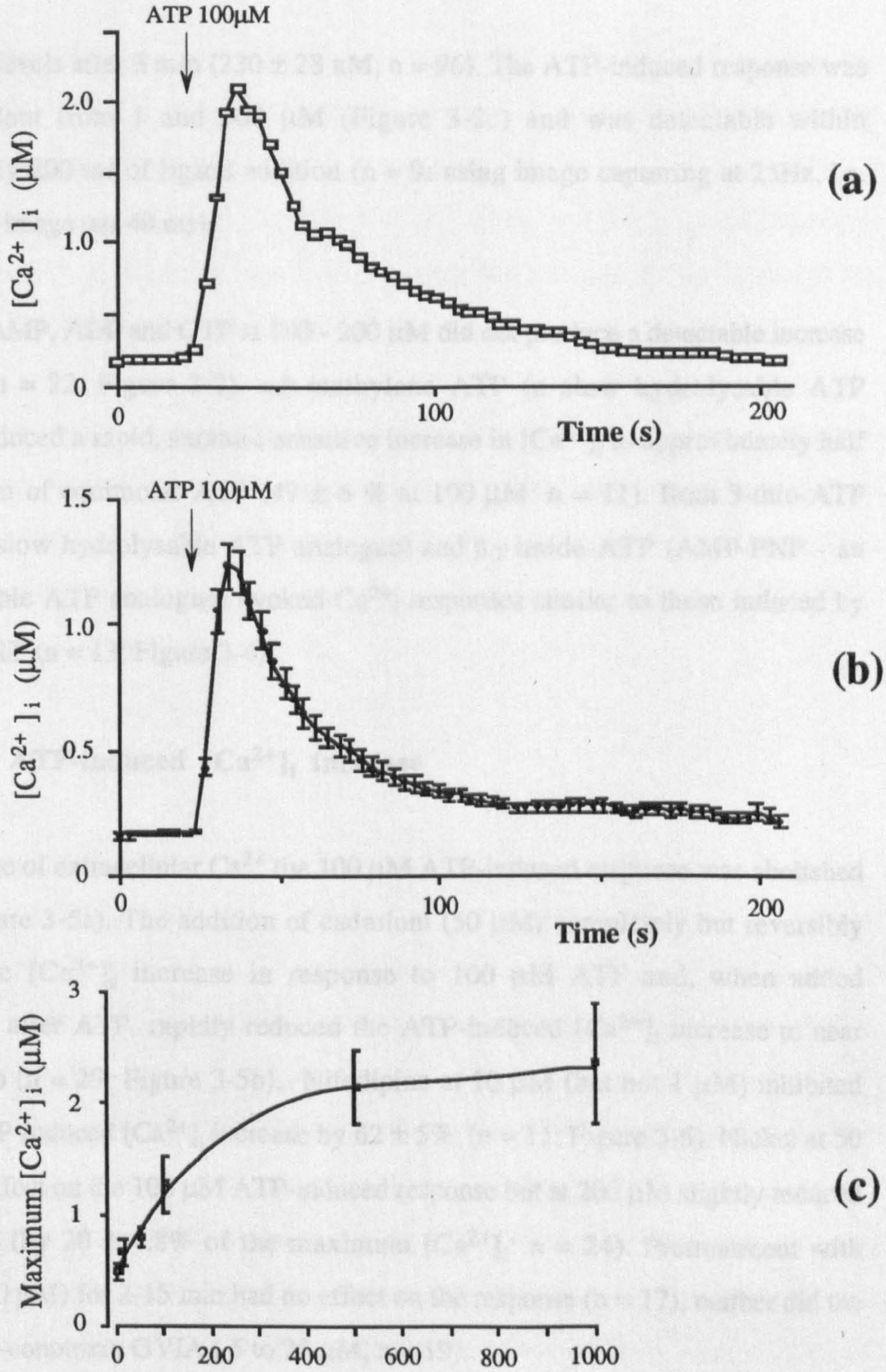


Figure 3-2. The effects of ATP on $[Ca^{2+}]i$. (a) Showing a typical $Ca^{2+}i$ recording from a single hypothalamic cell exposed to 100 μM ATP. The addition of ATP is indicated by the arrow. (b) The temporal effect of ATP (100 μM) on $[Ca^{2+}]i$ in rat hypothalamic neurons (mean ± SE: n = 96). The introduction of ATP is indicated by the arrow. (c) ATP dose/response curve (mean ± S.E: n = 11). Cells were exposed consecutively to 1, 10, 50, 100, 500 and 1000 μM ATP for 3 min at each concentration with 30 min washouts between ATP applications. The resting $[Ca^{2+}]i$ is 141 ± 26 (n = 11).

near resting levels after 3 min (230 ± 28 nM, n = 96). The ATP-induced response was dose-dependent from 1 and 500 μM (Figure 3-2c) and was detectable within approximately 200 ms of ligand addition (n = 9: using image capturing at 25Hz, i.e. one frame of image per 40 ms).

Adenosine, AMP, ADP and GTP at 100 - 200 μM did not produce a detectable increase in $[Ca^{2+}]_i$ (n = 22: Figure 3-3). α,β-methylene ATP (a slow hydrolysable ATP analogue) induced a rapid, suramin-sensitive increase in $[Ca^{2+}]_i$ to approximately half the maximum of equimolar ATP (47 ± 6 % at 100 μM: n = 11). Both 3-thio-ATP (ATPγS - a slow hydrolysable ATP analogue) and β,γ-imido-ATP (AMP-PNP - an unhydrolyzable ATP analogue) evoked Ca^{2+}_i responses similar to those induced by equimolar ATP (n = 13: Figure 3-4).

Sources of ATP-induced $[Ca^{2+}]_i$ increase

In the absence of extracellular Ca^{2+} the 100 μM ATP-induced response was abolished (n = 20: Figure 3-5a). The addition of cadmium (50 μM) completely but reversibly inhibited the $[Ca^{2+}]_i$ increase in response to 100 μM ATP and, when added immediately after ATP, rapidly reduced the ATP-induced $[Ca^{2+}]_i$ increase to near resting levels (n = 29: Figure 3-5b). Nifedipine at 10 μM (but not 1 μM) inhibited maximal ATP-induced $[Ca^{2+}]_i$ increase by 62 ± 5% (n = 11: Figure 3-6). Nickel at 50 μM had no effect on the 100 μM ATP-induced response but at 200 μM slightly reduced the response (by 20 ± 4.8% of the maximum $[Ca^{2+}]_i$: n = 24). Pretreatment with ryanodine (50 μM) for 2-15 min had no effect on the response (n = 17), neither did the addition of ω-conotoxin GVIA (5 to 75 μM, n = 19).

Effect of suramin and other neurotransmitter receptor antagonists on ATP-induced Ca^{2+}_i response

Suramin, a P_2-purinoceptor antagonist, had no affect on resting $[Ca^{2+}]_i$ in

hypothalamic neurons but reversibly inhibited the ATP-induced $Ca^{2+}{}_i$ response in a dose-dependent manner (ID_{50} = 25 μM: Figure 3-7a). At a concentration of 100 μM, suramin almost abolished the effect of equimolar ATP (n = 35: Figure 3-7b). In contrast, pretreatment with suramin had no effect on 5-HT-, acetylcholine-, noradrenaline- or glutamate-receptor agonists-induced increase in $[Ca^{2+}]_i$ in cultured hypothalamic neurons (n = 4 ~ 24: Figure 3-8).

Atropine, hexamethonium, ketanserin, bicuculline, DL-2-amino-5-phosphonovaleric acid (AP-5, an NMDA glutamate receptor antagonist), 6,7-dinitroquinoxaline-2,3-dione (DNQX, non-NMDA glutamate receptor antagonist) and 8-cyclopentyltheophylline (CPDMX, adenosine receptor antagonist) did not significantly affect the ATP-induced $Ca^{2+}{}_i$ increase (n = 9 ~ 19: Figure 3-9).

Discussion

Extracellular ATP-provoked intracellular Ca^{2+} responses in cultured hypothalamic neurons are highly specific, as: 1) adenosine, AMP, ADP and GTP failed to elicit a response (Figure 3-3); 2) antagonists of muscarinic, nicotinic, NMDA, non-NMDA, GABA, 5HT and adenosine receptors had no effects on the ATP-induced $Ca^{2+}{}_i$ responses (Figure 3-9); 3) the ATP-induced $Ca^{2+}{}_i$ responses were blocked by suramin (Figure 3-7), a P_2-purinoceptor antagonist (Dunn & Blakeley 1988, von Kügelgen *et al.* 1990, Hoyle *et al.* 1990, Nakazawa *et al.* 1990b); and 4) suramin had no effect on 5-HT-, acetylcholine-, noradrenaline- or glutamate-receptor agonists-induced increase in $[Ca^{2+}]_i$ (Figure 3-8). These results pharmacologically demonstrated the presence of a receptor specific for ATP in cultured hypothalamic neurons.

The present study indicates that only a subpopulation of hypothalamic neurons express ATP receptors since an ATP-induced Ca^{2+} response occurred in 42% of hypothalamic neurons examined. At the end of each experiment all cells were shown to respond to

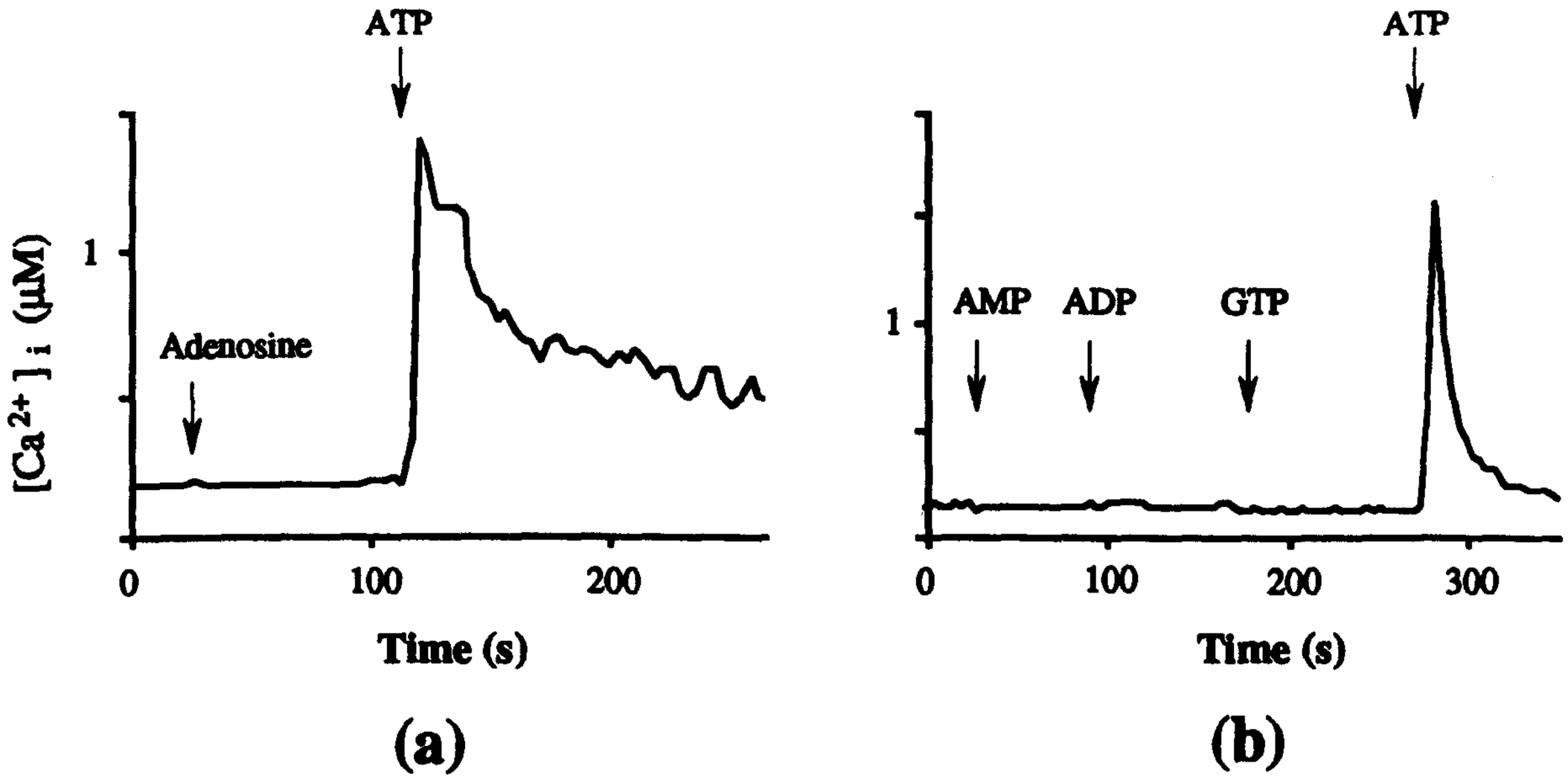


Figure 3-3. Effects of adenosine and other nucleotides on $[Ca^{2+}]i$ in ATP-responsive cells. Representative recordings were made from two individual cells: 100 μM ATP was used. (a) The absence of $Ca^{2+}i$ response to adenosine (200 μM). (b) The absence of $Ca^{2+}i$ response to AMP, ADP and GTP (all 100 μM). The ligand additions are indicated by the arrows.

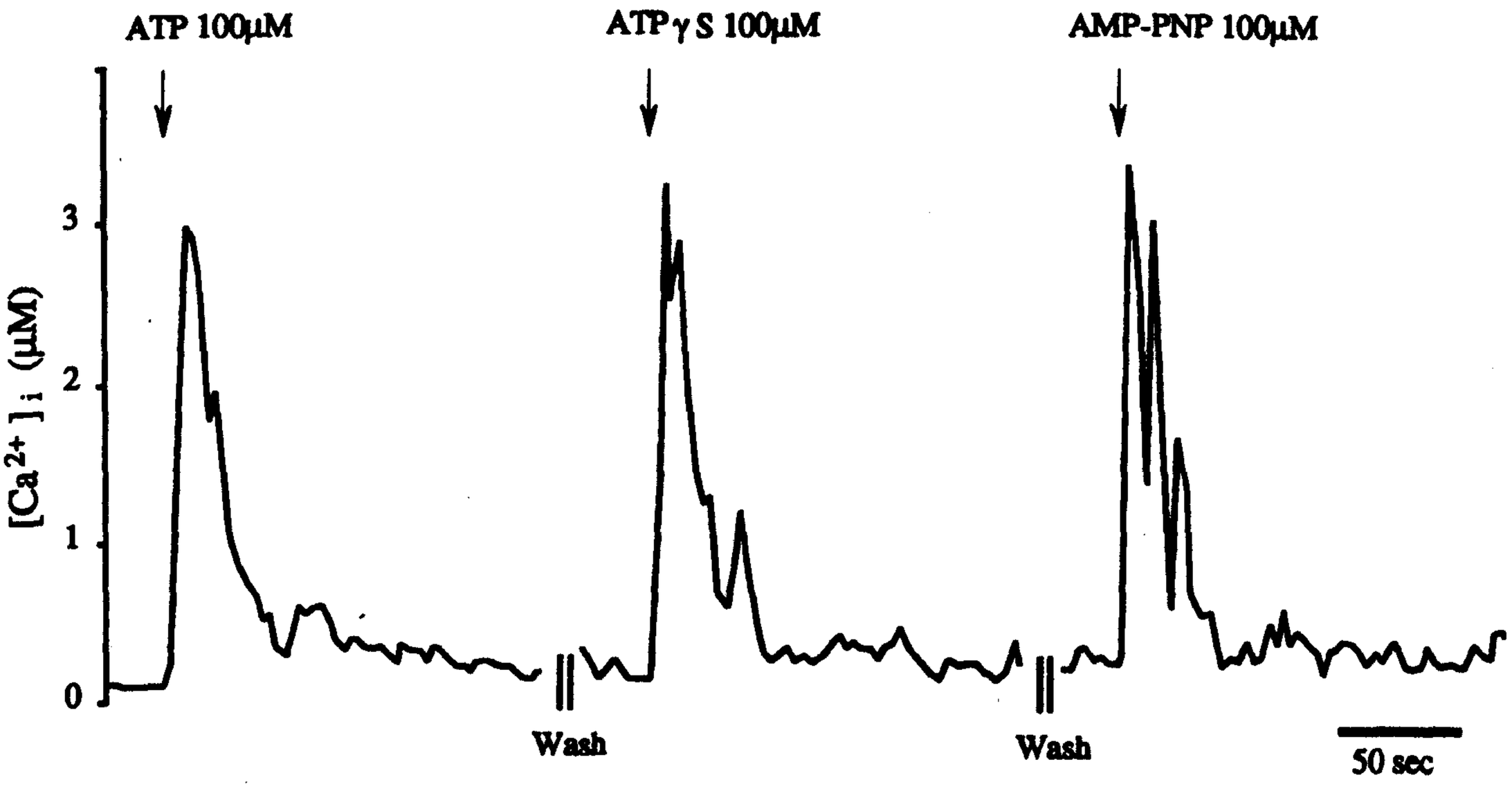
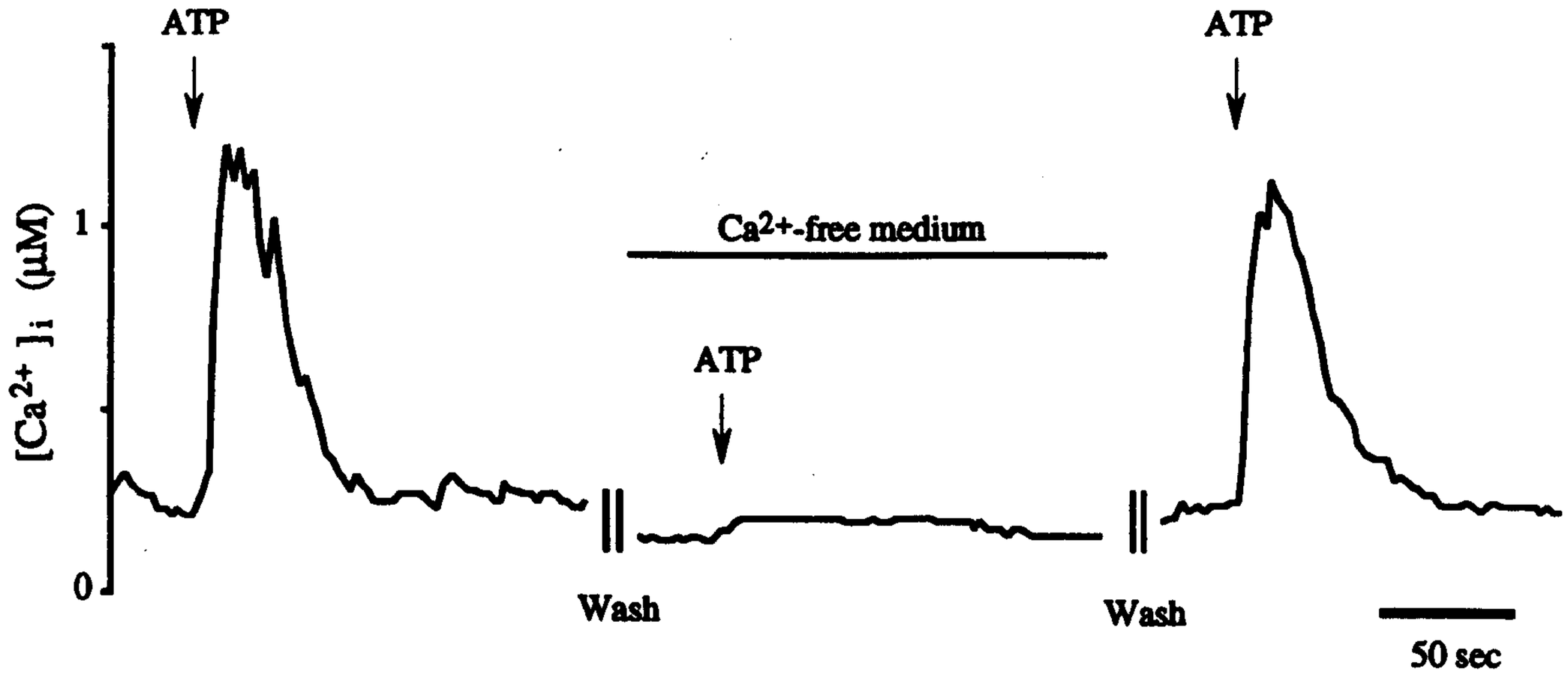
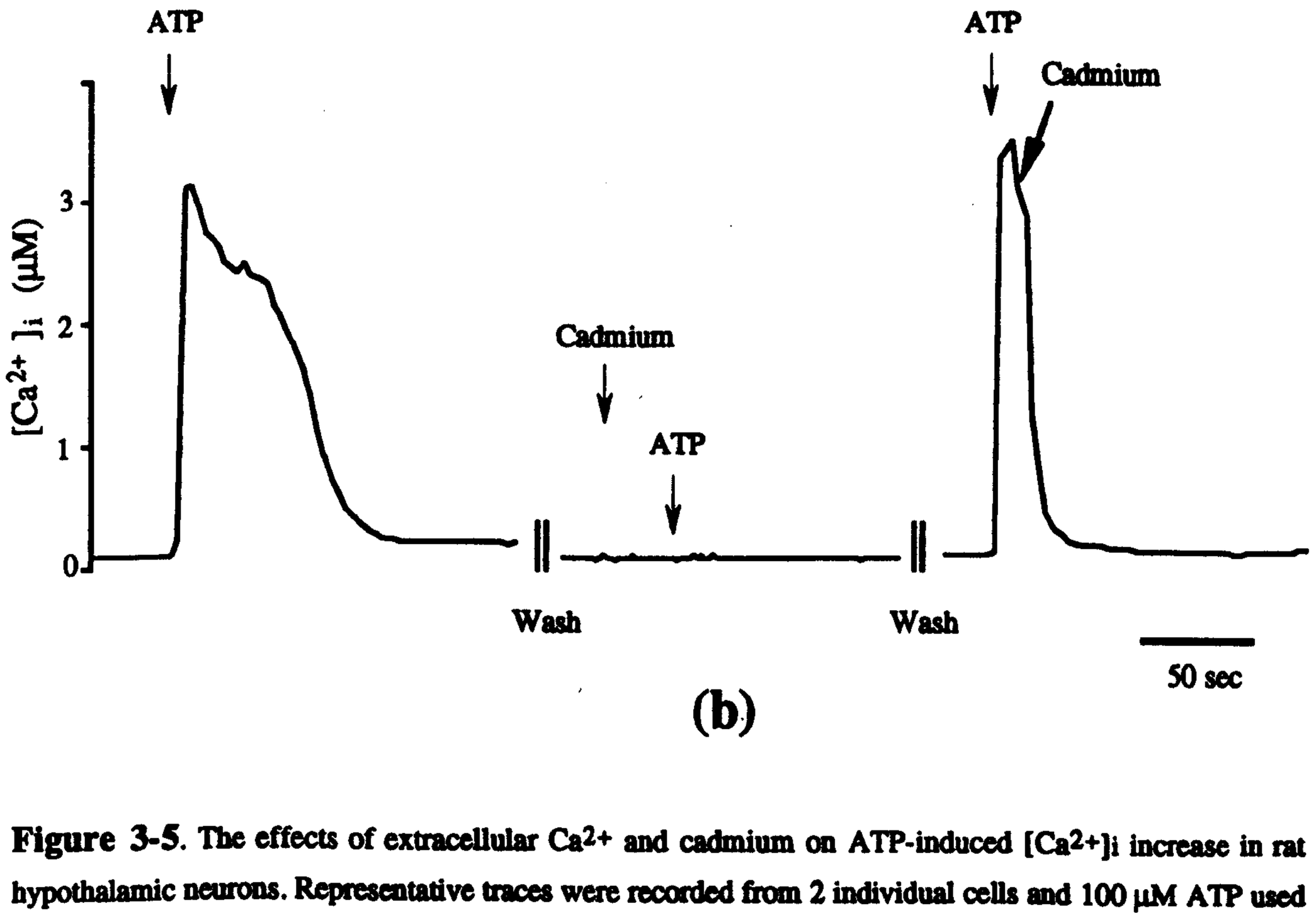


Figure 3-4. Effects of ATP analogues on $[Ca^{2+}]i$. A representative recording from a single hypothalamic neuron exposed sequentially to ATP, 3-thio-ATP (ATPγS) and β,γ-imido-ATP (AMP-PNP) with 30 min washouts between recordings. The point of ligand additions are indicated by the arrows.



(a)



(b)

Figure 3-5. The effects of extracellular Ca^{2+} and cadmium on ATP-induced $[Ca^{2+}]i$ increase in rat hypothalamic neurons. Representative traces were recorded from 2 individual cells and 100 μM ATP used with 30 min washouts between recordings. (a) Following removal of extracellular Ca^{2+} by perfusion with Ca^{2+}-free, 0.1 mM EGTA-containing buffer for 5 min, the ATP-induced $[Ca^{2+}]i$ increase was abolished. (b) The addition of cadmium (50 μM) completely but reversibly blocked the $Ca^{2+}i$ response to ATP, and when added after ATP stimulation, accelerated $[Ca^{2+}]i$ return to baseline (b: right hand panel). The additions of compounds are indicated by arrows.

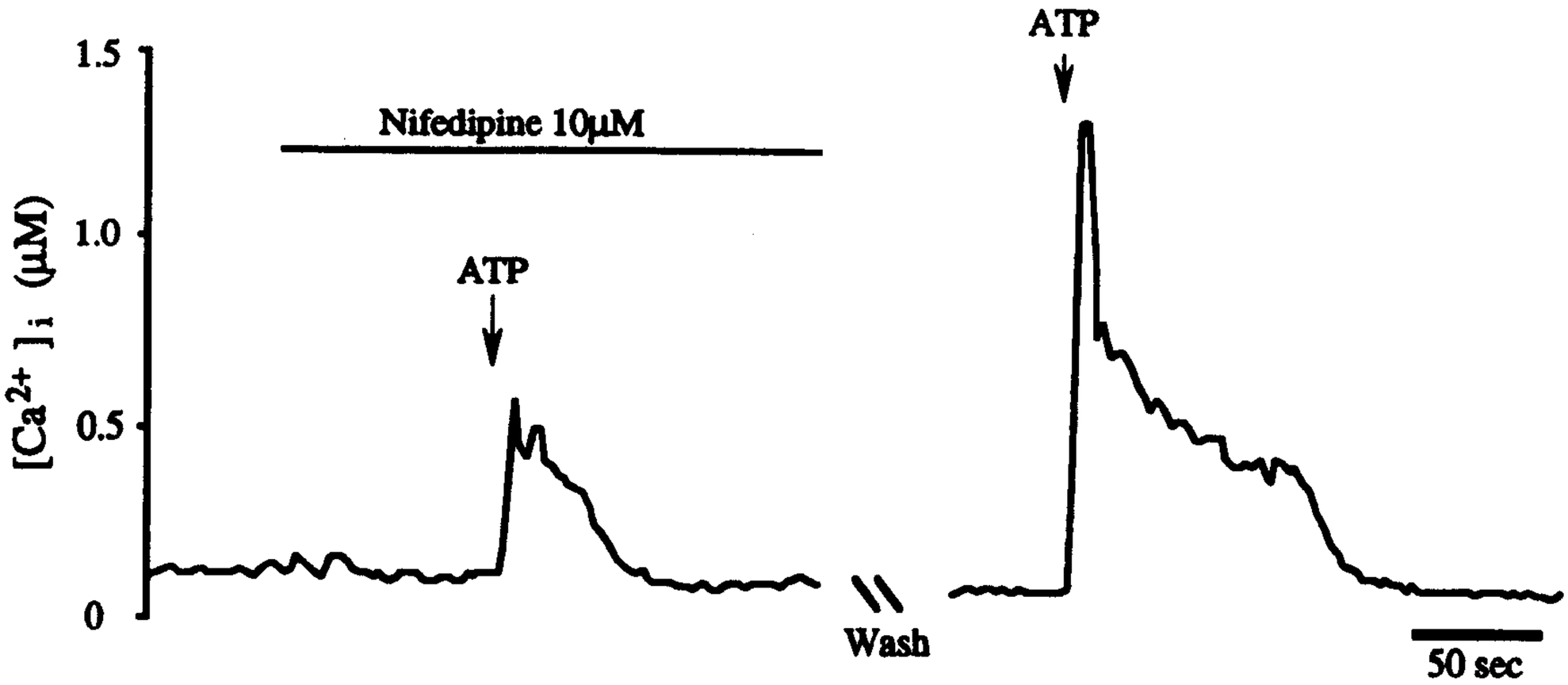
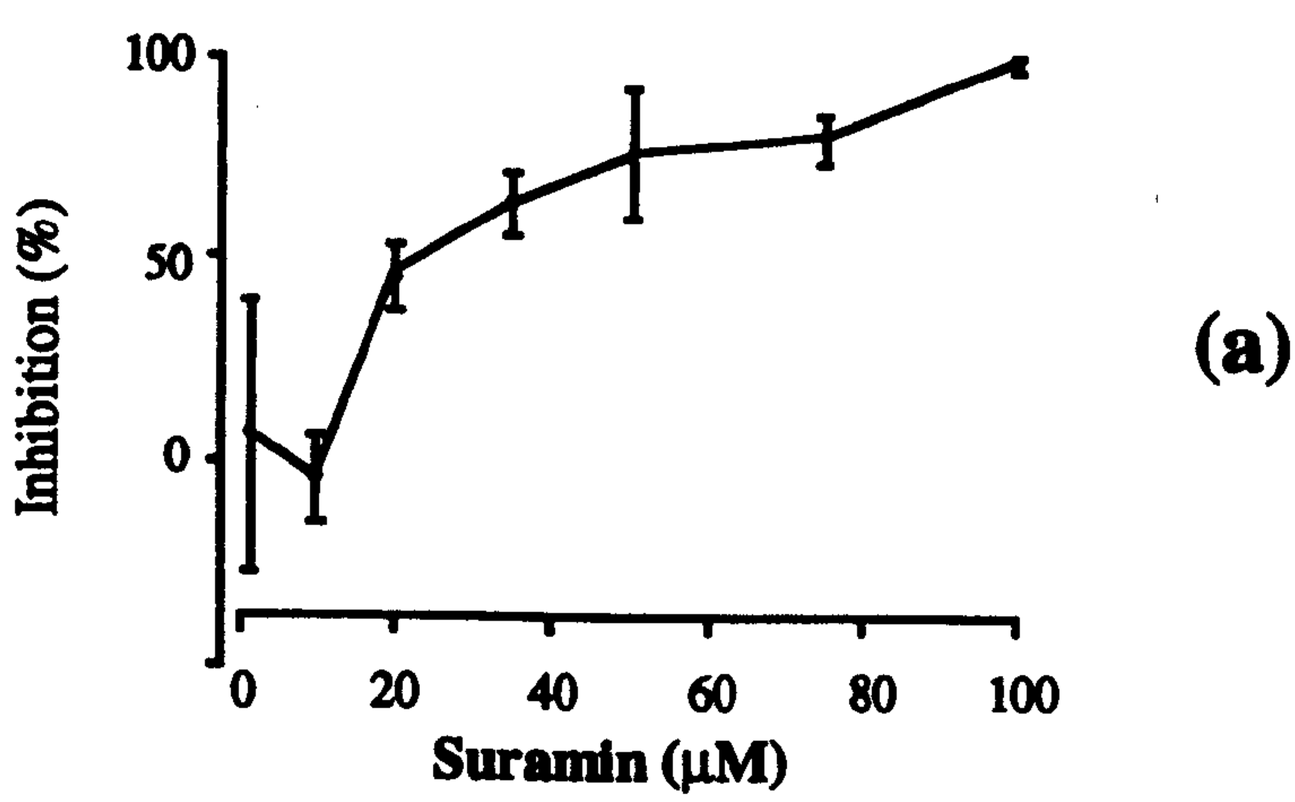
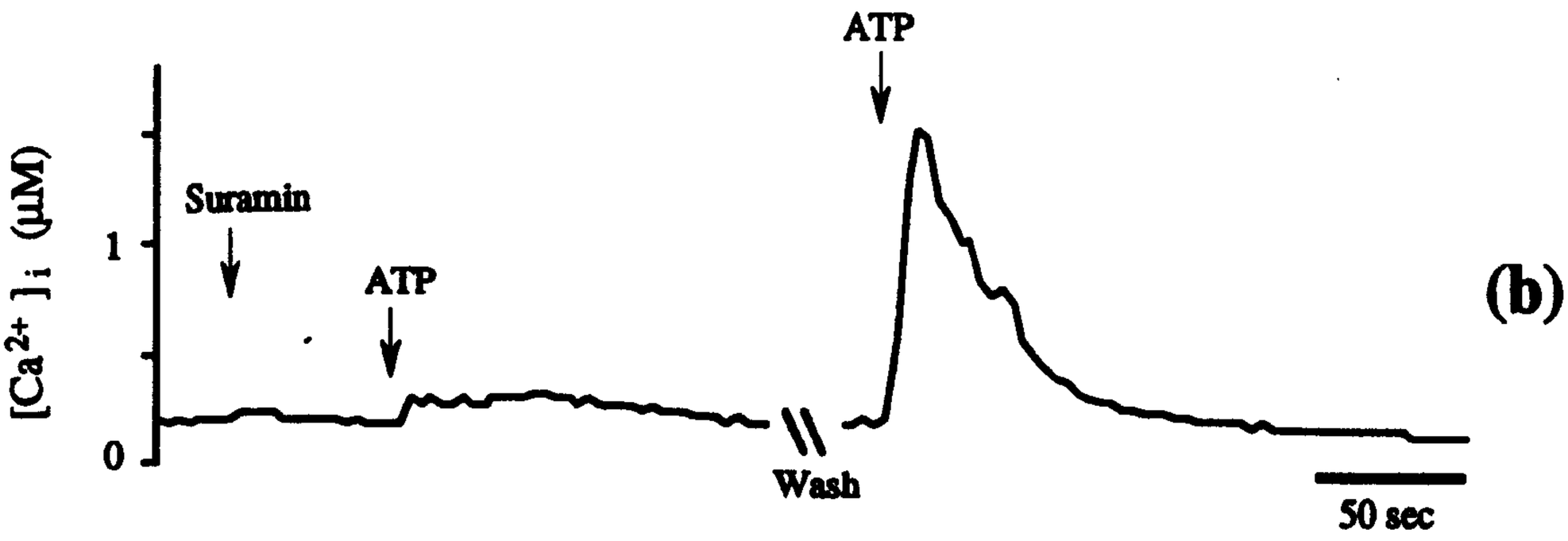


Figure 3-6. A representative example of the inhibitory effect of nifedipine on ATP (100 μM)-induced $[Ca^{2+}]i$ increase in a hypothalamic cell with 30 min washouts between recordings. The additions of compounds are indicated by the arrows.



(a)



(b)

Figure 3-7. (a) Dose-dependent inhibition of ATP (100 μM)-induced $Ca^{2+}i$ response by suramin (mean ± SE: n ≥ 13). (b) Suramin (100 μM)-induced reversible inhibition of the $Ca^{2+}i$ response to 100μM ATP in a single rat hypothalamic neuron. A 30 min washout separates the recordings. The additions of ligands are indicated by arrow.

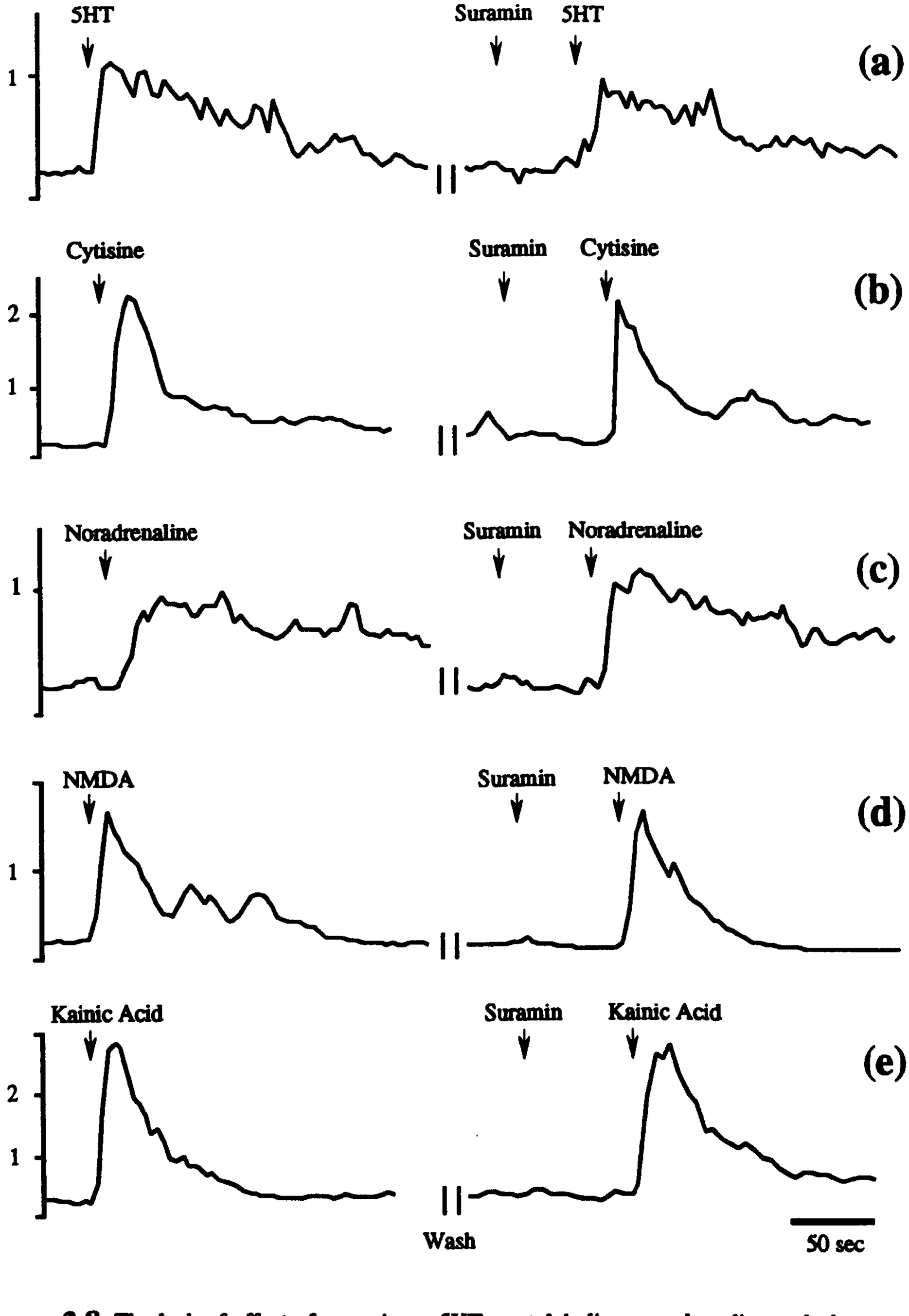


Figure 3-8. The lack of effect of suramin on 5HT, acetylcholine, noradrenaline and glutamate receptor activity in rat hypothalamic cells. Representative traces were recorded from 5 individual cells (a-e). The same agonists were applied twice, firstly in the absence of and subsequently in the presence of 100 μM suramin with 30 min washouts between recordings. The concentrations of agonists used were: (a) 5HT 100 μM, (b) cytisine 10 μM (nicotinic receptor agonist), (c) noradrenaline 100 μM, (d) NMDA 10 μM, & (e) kainic acid 1 μM (non-NMDA receptor agonist). The points of addition of ligands are indicated by the arrows.

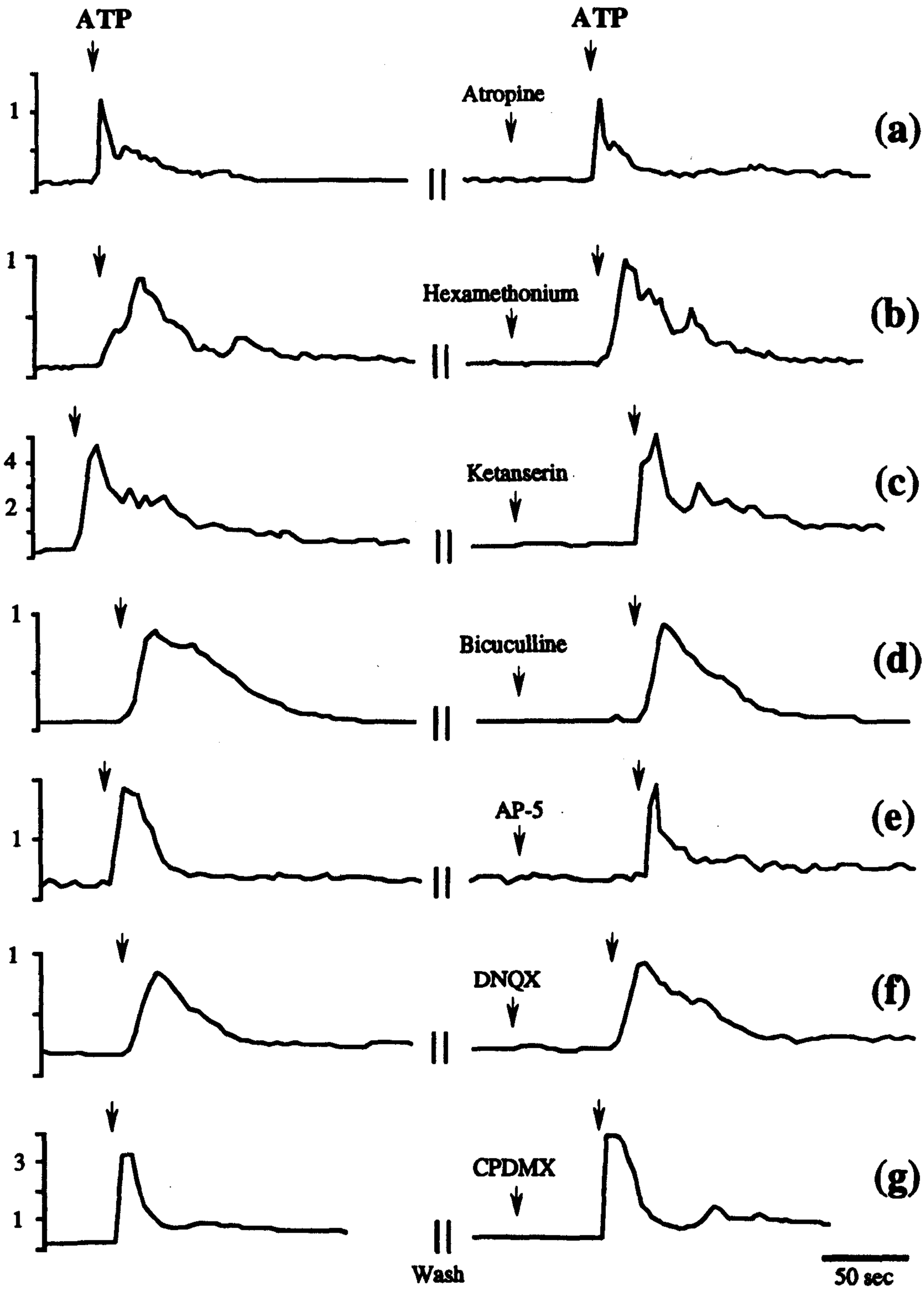


Figure 3-9. The lack of effect of acetylcholine, 5HT, GABA, glutamate and adenosine receptor antagonists on ATP-induced $Ca^{2+}i$ response in rat hypothalamic cells. Representative traces were obtained from 7 individual cells (a-g). ATP (100 μM) was applied twice, firstly in the absence of and subsequently in the presence of the various antagonists with 30 min washouts between recordings. Doses used were: (a) atropine 100 μM; (b) hexamethonium 100 μM; (c) ketanserin 1 μM; (d) bicuculline 50 μM; (e) DL-2-amino-5-phosphonovaleric acid (AP-5) 100 μM; (f) 6,7-dinitroquinoxaline-2,3-dione (DNQX) 100 μM; and (g) 8-cyclopentyltheophylline (CPDMX) 100 μM. The additions of the antagonists and ATP are indicated by arrows.

other neurotransmitters (such as NMDA and kainic acid) and to depolarization by high concentration of K^+, excluding impaired cell viability as an explanation for response failure. The work from other investigators showed similar ATP-responsive subpopulation in vertebrate sensory neurons, hippocampal neurons and intracardiac neurons (Jahr & Jessell 1983, Krishtal *et al.* 1983, Bean 1990, Allen & Burnstock 1990, Inoue *et al.* 1992).

As the observed ATP-induced $[Ca^{2+}]_i$ increase was abolished by both removal of extracellular Ca^{2+} and by cadmium, which blocks high voltage-activated (HVA) Ca^{2+} channels, Ca^{2+} influx appears to be responsible for the ATP-induced $[Ca^{2+}]_i$ increase in rat hypothalamic neurons. This is further supported by the lack of effect of ryanodine, an intracellular Ca^{2+} release inhibitor, on ATP-induced $[Ca^{2+}]_i$ increase. Electrophysiological experiments have shown that HVA Ca^{2+} currents in rat hypothalamic neurons are completely blocked by 10 μM cadmium (Müller *et al.* 1992), whereas ATP-gated Ca^{2+} currents in muscle cells are unaffected by up to 500 μM cadmium (Benham & Tsien 1987, Thomas & Hume 1990) (although they can be blocked at much higher levels (5 mM) (Thomas & Hume 1990)). In contrast, low voltage-activated (LVA) Ca^{2+} currents in hypothalamic neurons are strongly inhibited by 50 μM nickel (Müller *et al.* 1992). In the present study, complete inhibition of ATP-induced Ca^{2+} response by 50 μM cadmium with 20% inhibition by nickel suggests that Ca^{2+} entry through HVA channels is most likely to be responsible for the observed ATP-induced $[Ca^{2+}]_i$ increase in rat hypothalamic neurons. The lack of effect of ω-conotoxin GVIA, and inhibition of ATP-induced $[Ca^{2+}]_i$ increase by nifedipine (albeit by only 62% at 10 μM - in keeping with the findings from electrophysiological experiments (Müller *et al.* 1992)) suggests that 'L'- rather than 'N'- type HVA Ca^{2+} channels predominate. The present findings of intracellular Ca^{2+} signaling in hypothalamic neurons are similar to those observed in cardiac myocytes (Danziger *et al.* 1988, De Young & Scarpa 1989, Christie *et al.* 1992), but differs from the findings in a neuroblastoma cell line in which mobilization of Ca^{2+} solely from intracellular stores is responsible for an increase in $[Ca^{2+}]_i$ (Iredale *et al.* 1992a,

Iredale *et al.* 1992b).

As the observed Ca^{2+} response in hypothalamic neurons was rapid, which could be seen within approximately 200 ms after ligand addition, and no evidence was found in this study to indicate the involvement of intracellular Ca^{2+} mobilization from intracellular Ca^{2+} stores, the ATP receptor found in hypothalamic neurons appears to fall into the ATP-gated ion channel subtype of P_2 purinoceptor (i.e. P_{2X} subtype). Because a full range of ATP receptor agonists has not been tested in this study, the subtype of this receptor is not clear. As discussed above, the ATP-induced increase in $[Ca^{2+}]_i$ in hypothalamic neurons is caused by Ca^{2+} influx via Ca^{2+} channels. It seems that activation of these ATP receptors lead to cell depolarization and this membrane depolarization then causes opening of Ca^{2+} channels.

The permeability of ATP-gated channels to cation varies with cell types. In both arterial muscle cells (Benham & Tsien 1987) and rat nucleus solitarii neurons (Ueno *et al.* 1992a) Ca^{2+} is more permeable in ATP-activated channels than Na^+, while in sensory neurons (Jahr & Jessell 1983, Krishtal *et al.* 1988, Bean *et al.* 1990) Na^+ is a predominant ion through ATP-activated channels. Inferred from the present study based on intracellular Ca^{2+} imaging, the hypothalamic ATP receptor appears not to be very permeable to Ca^{2+}. The present study is, however, unable to rule out a contribution to the increase in $[Ca^{2+}]_i$ by Ca^{2+} entry through ATP-activated ion channels.

It has been proposed that protein phosphorylation by ecto-protein kinase might initiate the ATP-induced response (Zhang *et al.* 1988, Ehrlich *et al.* 1990, Christie *et al.* 1992). This would imply that hydrolysable ATP should be required for ATP-induced responses. The present results, together with the findings of others (Benham & Tsien 1987, Iredale *et al.* 1992a, Sun *et al.* 1992, Weiss *et al.* 1992), however, do not support this view as the very slowly hydrolysable ATP analogue ATPγS and the non-hydrolysable analogue β,γ-imido-ATP (AMP-PNP) were as efficient as ATP in eliciting

currents or a $[Ca^{2+}]_i$ increase (Figure 3-4).

Chapter 4. PITUITARY ATP RECEPTORS: PHARMACOLOGICAL CHARACTERIZATION, FUNCTIONAL LOCALIZATION, CLONING AND SEQUENCING

Introduction

Extracellular ATP is now known to mediate a variety of responses in a number of biological systems via ATP receptors (P_2 purinoceptors). In pituitary cell cultures, ATP was shown to strongly stimulate inositol phosphate accumulation (Davidson *et al.* 1990) and this effect appeared to be mediated by P_{2U} subtype of ATP receptors. No significant pituitary hormone release in response to ATP was, however, observed in this early investigation. Thus, it was concluded that none of the function-specific pituitary cell types, i.e. lactotropes, somatotropes, gonadotropes, corticotropes or thyrotropes, was direct targets for ATP action. Although this conclusion is logical from this data, it renders difficult the interpretation of the ATP-induced inositol phosphate signaling, which was actually much stronger than that induced by specific releasing hormones such as thyrotropin-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH). Considering the limitation of the techniques employed by this early investigation, the present study was to re-examine ATP receptors in pituitary cells using sensitive modern techniques such as real-time calcium ion imaging of Fura-2 fluorescence at a single cell level and molecular cloning.

Methods

Primary pituitary cells were cultured from adult female Wistar rats (approximately 150 g). After 1 day and after 2 to 3 days in culture rat pituitary cells and αT3-1 cells, respectively, were loaded with 2 μM Fura-2 AM at 37°C for 30 min and real time digital calcium ion imaging was performed at 35°C. Cells were exposed to ligand by rapidly pipetting 2.5 ml of medium containing the stimulus into the recording chamber which was maintained at approximately 0.5 ml by means of an aspiration tube positioned 2

mm above the coverslip. Cells with a clear Ca^{2+} response (i.e. those in which stimulation at least doubled the $[Ca^{2+}]_i$) were defined as 'ligand-responsive' and used to generate the data shown. All experiments were repeated on at least three occasions and data shown are either from a single representative experiment or are pooled from multiple experiments as described.

Total RNA was extracted from adult Wistar rats of either sex and molecular cloning was performed as described in Chapter 2.

Results

Intracellular Ca^{2+} Responses

ATP (100 μM) rapidly increases cytosolic Ca^{2+} in approximately 30% of 1765 rat pituitary cells in primary culture. GnRH (100 nM) increased $[Ca^{2+}]_i$ in 9% of the cells in this heterogeneous population and of these, 82% also responded to ATP (10 to 100 μM). The cells which responded to ATP and GnRH were not responsive to corticotropin-releasing hormone (CRH), growth hormone releasing hormone (GRH) and TRH (Figure 4-1a). Thus, it is clear that GnRH responsive gonadotropes are responsive to ATP. This identification of gonadotropes as major targets for ATP action is further confirmed by studies on cells from the gonadotrope derived αT3-1 cell line (Windle *et al.* 1990, McArdle *et al.* 1992) which respond both to GnRH and ATP as described below.

In gonadotropes, 100 μM ATP induced a rapid 7-fold increase in $[Ca^{2+}]_i$ from a baseline of 132 ± 20 nM to the maximum of 896 ± 95 nM (Mean ± SE: n = 28 previously unstimulated cells), followed by a plateau phase, returning from 200 - 500 nM to baseline over approximately 4 minutes. The $Ca^{2+}{}_i$ response was concentration-dependent between 0.1 and 100 μM of ATP (Figure 4-1b), and in the absence of extracellular Ca^{2+} the plateau phase only was attenuated (Figure 4-1c), indicating that Ca^{2+} mobilization from intracellular pools is responsible for the initial ATP-induced

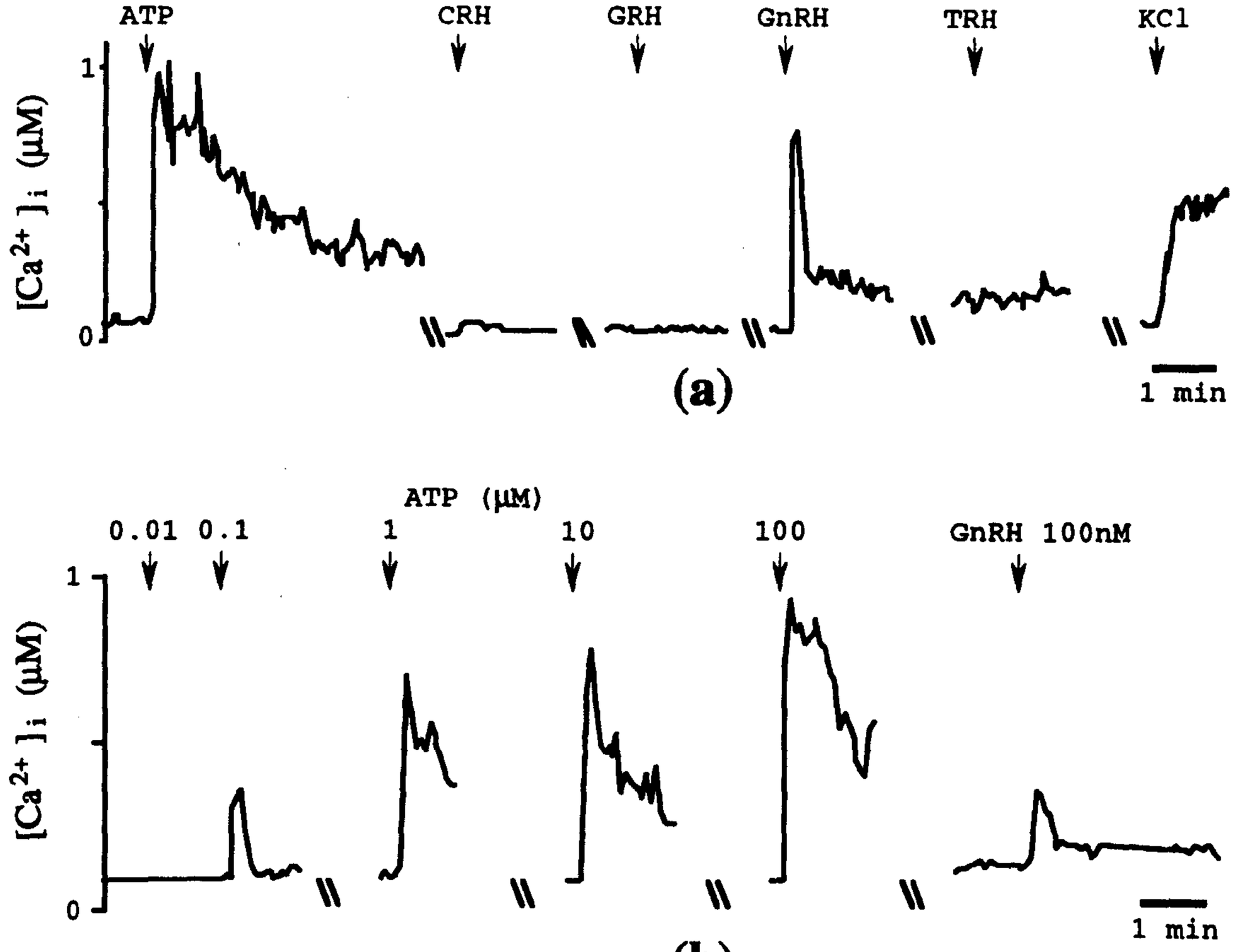


Figure 4-1. ATP-induced cytosolic Ca^{2+} responses in gonadotropes. (a) Representative recording showing the effect of ATP on cytosolic Ca^{2+} in a single rat pituitary cell functionally characterized as a gonadotrope. The successive stimulations with ATP (100 μM), CRH (100 nM), GRH (100 nM), GnRH (100 nM), TRH (100 nM) and KCl (40 mM) were separated by washouts periods of 2 - 30 min. (b) Concentration dependence of the ATP-induced Ca^{2+} response in a gonadotrope successively stimulated with 0.01-100 μM ATP. Each washout period between the stimulations was 20 min. (c) Recording from a single gonadotrope showing intracellular Ca2+ mobilization. The cell was stimulated first in Ca^{2+}-free medium containing 0.1 mM EGTA as indicated by the bar and then in normal Ca^{2+}-containing medium. 20 min of washouts separated the stimulations.

Ca^{2+}_i spike. A similar Ca^{2+}_i response was produced by equimolar UTP and ADP, whereas UDP (100 μM) caused a modest increase in $[Ca^{2+}]_i$ and GTP (100 μM) had only a very small stimulatory effect. Adenosine, AMP, GDP, UMP, CDP and CTP (all at 100 μM) were ineffective.

Suramin, a selective P_2-purinoceptor antagonist, had no effect on GnRH-induced $[Ca^{2+}]_i$ increase but reversibly reduced the maximal ATP-induced $[Ca^{2+}]_i$ response in gonadotropes (Figure 4-2a) and αT3-1 cells (not shown) by approximately 40% (mean ± SEM: 41 ± 20%: n = 5 cells) and 60% (61 ± 5%: n = 43 cells), respectively. The P_{2X} receptor agonists β,γ-methylene ATP and α,β-methylene ATP (both 100 μM) did not increase cytosolic Ca^{2+} in gonadotropes (Figure 4-2b) or αT3-1 cells (not shown), whereas the P_{2Y} receptor agonist 2-methylthioATP had only a modest stimulatory effect in both cell types (Figure 4-2b and data not shown).

In αT3-1 cells, ATP, ADP and UTP all increased cytosolic Ca^{2+} with comparable potency and efficacy (Figure 4-3).

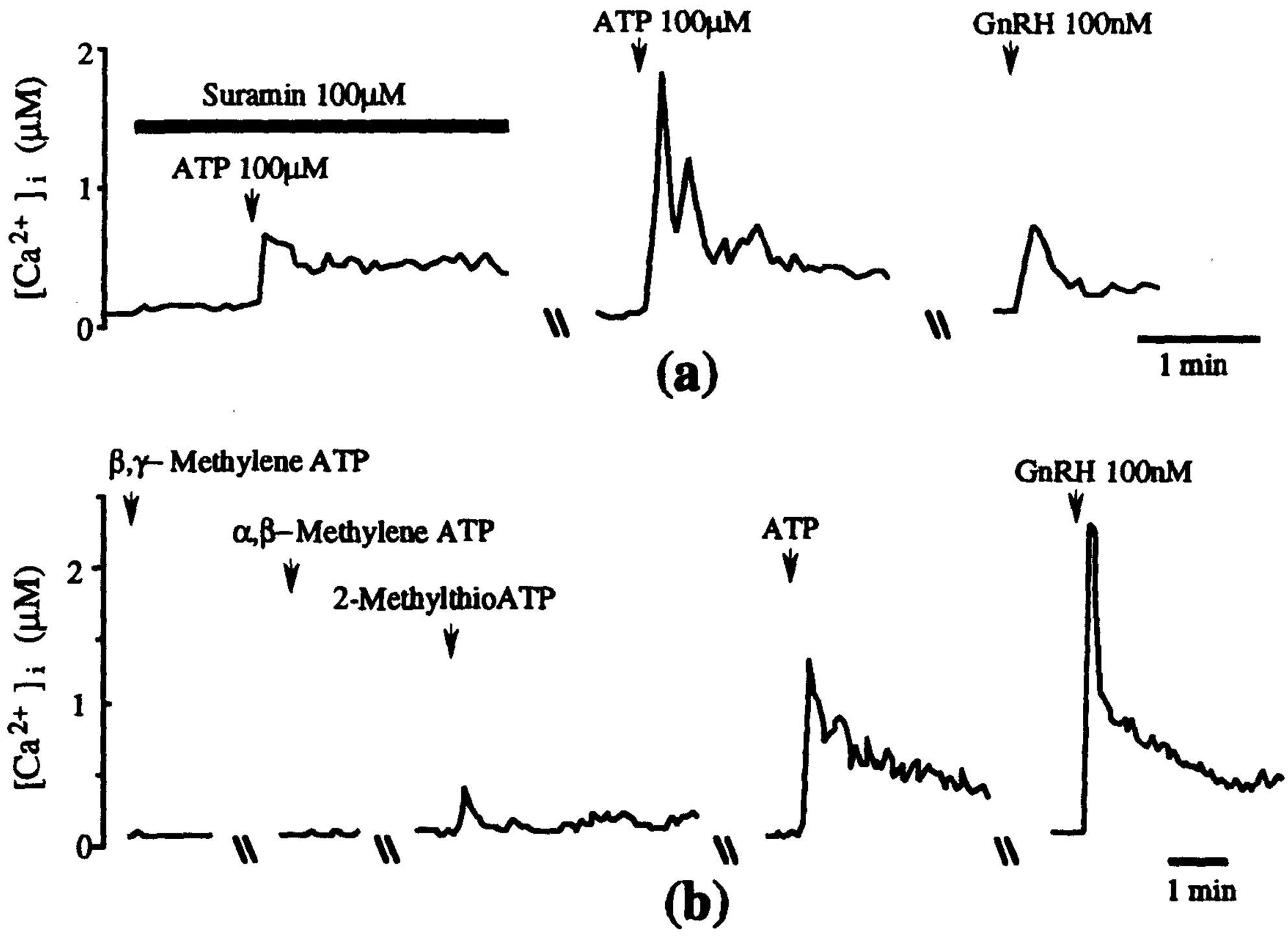


Figure 4-2. Effects of P2-purinoceptor antagonist and agonists on $[Ca^{2+}]i$ in gonadotropes. (a) Representative recording from a single gonadotrope showing the effect of the P2 receptor antagonist suramin on ATP-induced $Ca^{2+}i$ response. The application of suramin is indicated by the bar and the stimulations were separated by washouts periods of 20 min. (b) Representative recording from a single gonadotrope showing the effects of the P2X receptor agonists β,γ-methylene ATP (100 µM) and α,β-methylene ATP (100 µM), and the P2Y receptor agonist 2-methylthio ATP (100 µM). The stimulations were separated by washouts periods of 2 - 20 min.

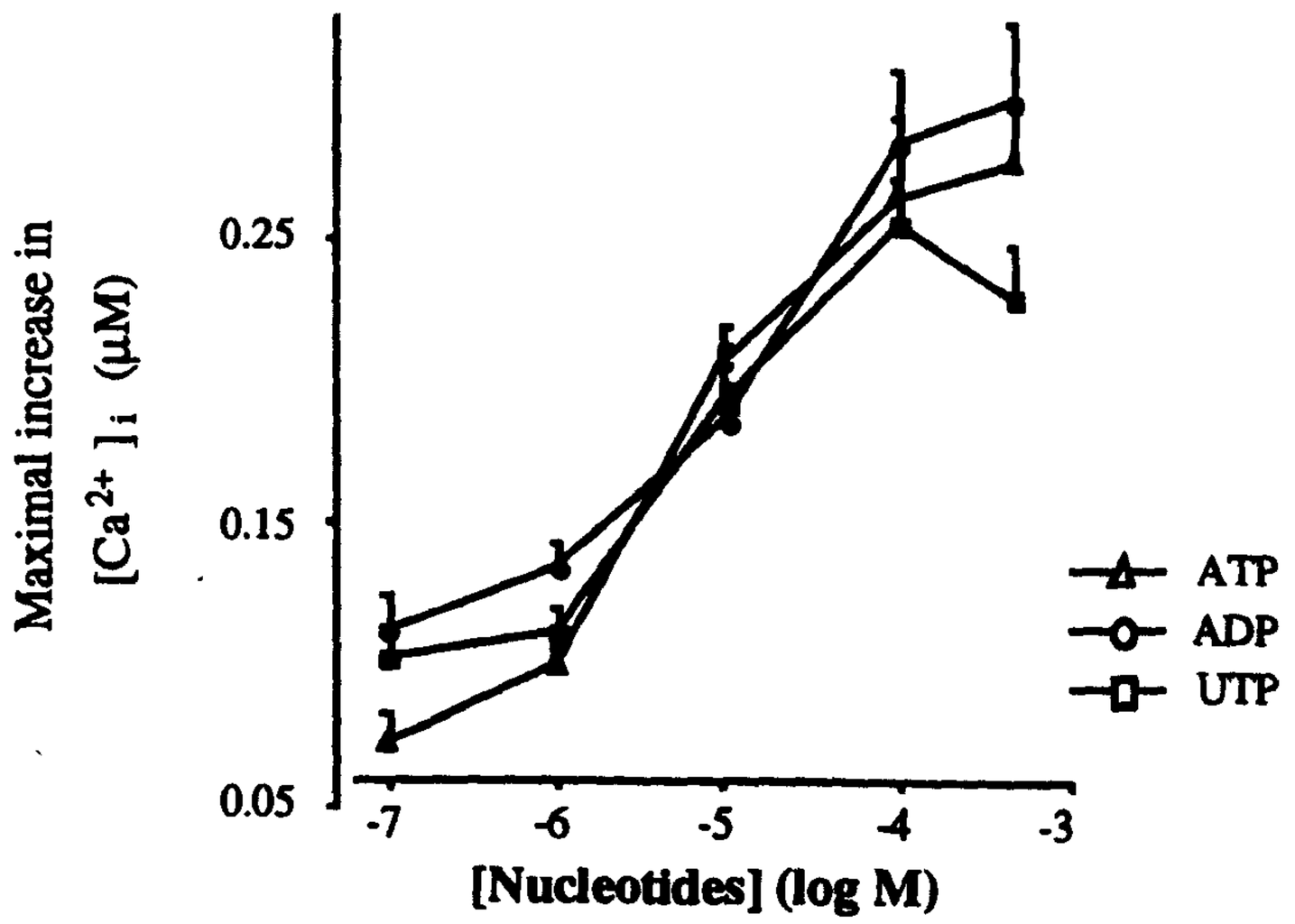


Figure 4-3. Concentration-response curves showing the maximal increase in $[Ca^{2+}]i$ (mean + SEM: n ≥ 15 cells from 3-5 separate experiments) in response to ATP, ADP and UTP with the indicated concentrations in previously unstimulated αT3-1 cells.

The simultaneous addition of 100 μM ATP plus 100 μM UTP failed to elicit a stronger Ca^{2+}_i response than either alone in normal gonadotropes or in αT3-1 cells (Figure 4-4). Pretreatment with ATP (100 μM, 4 min) considerably reduced the subsequent response to ATP or UTP and pretreatment with UTP similarly reduced subsequent responses to ATP or UTP (Figure 4-5), while the cells remained highly responsive to subsequent challenge with 10 nM GnRH, yielding a similar increase in $[Ca^{2+}]_i$ to that in unstimulated cells.

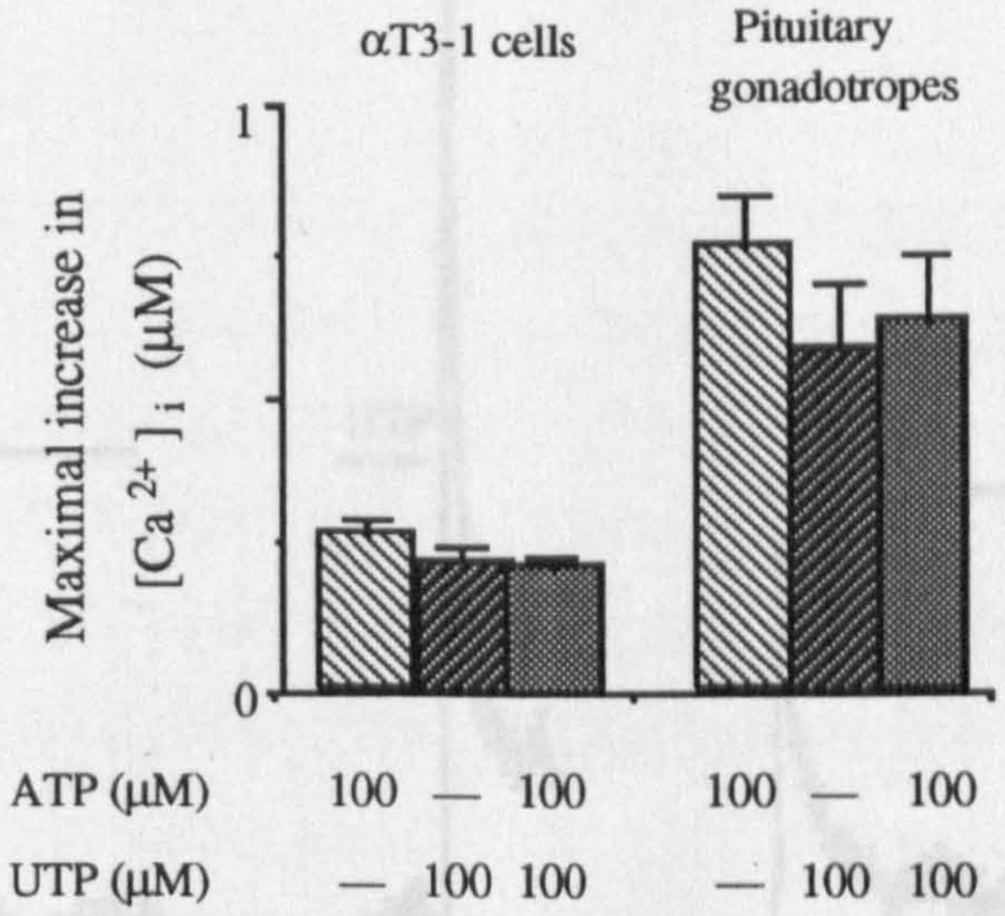


Figure 4-4. No additive effect of ATP and UTP at the maximal concentration on $[Ca^{2+}]i$. αT3-1 cells (left panel) and rat pituitary gonadotropes (right panel) were stimulated with ATP and UTP alone or in combination as indicated. The Figure shows pooled maximal increase in $[Ca^{2+}]i$ (mean ± SEM from ≥ 35 αT3-1 cells and ≥ 8 pituitary gonadotropes, respectively) in which the responses did not differ significantly from one another ($p > 0.05$, unpaired t-test).

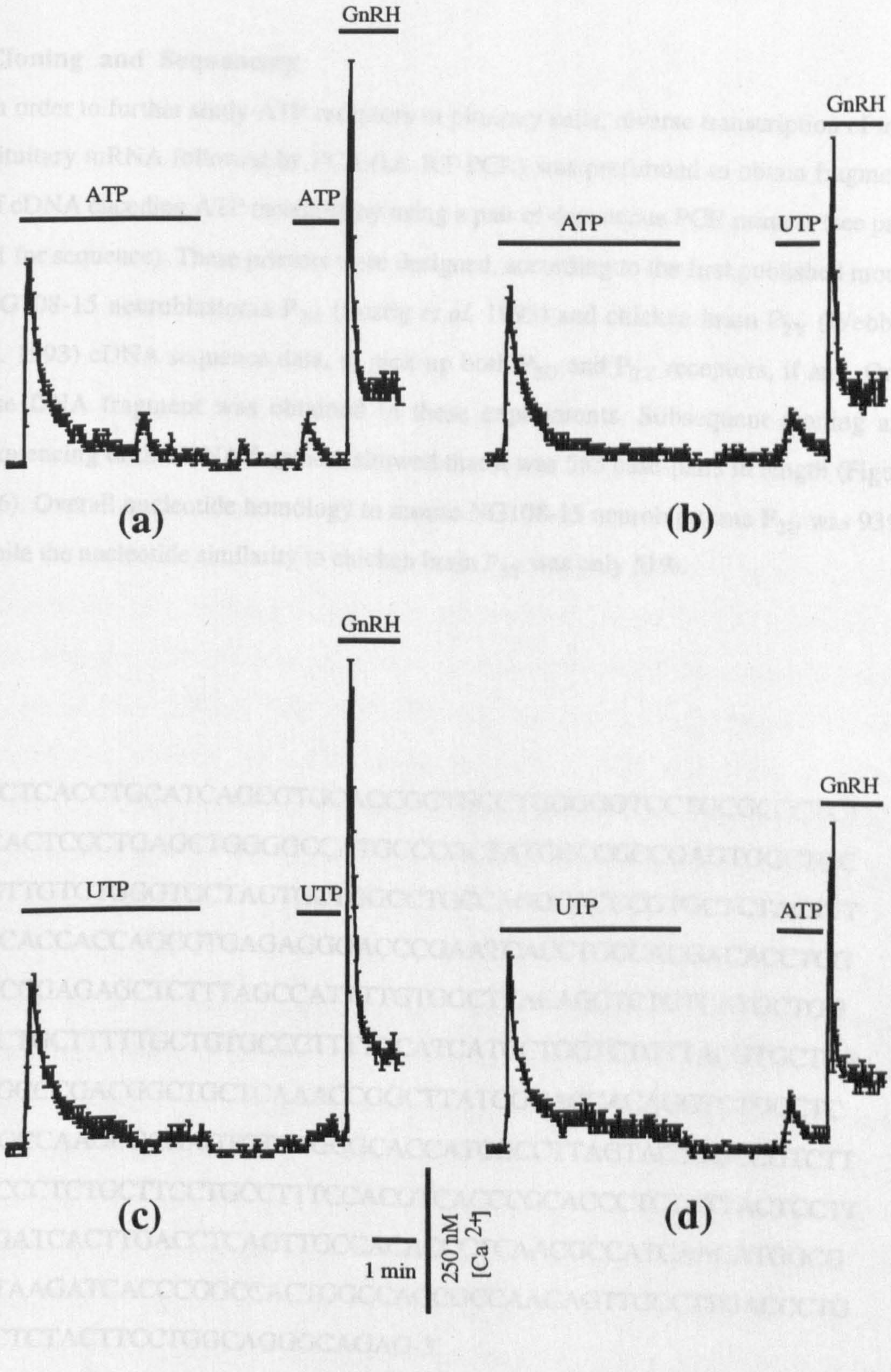


Figure 4-5. Homologous and heterologous desensitization of ATP and UTP effects in αT3-1 cells. Cells were stimulated as indicated with ATP, UTP (both 100 μM) and GnRH (10 nM). Each panel shows the mean ± SEM of $[Ca^{2+}]_i$ values in ≥ 8 cells (from a single representative experiment). The resting $[Ca^{2+}]_i$ was approximately 30 nM for all the experiments.

Cloning and Sequencing

In order to further study ATP receptors in pituitary cells, reverse transcription of total pituitary mRNA followed by PCR (i.e. RT-PCR) was performed to obtain fragments of cDNA encoding ATP receptors by using a pair of degenerate PCR primers (see page 61 for sequence). These primers were designed, according to the first published mouse NG108-15 neuroblastoma P_{2U} (Lustig *et al.* 1993) and chicken brain P_{2Y} (Webb *et al.* 1993) cDNA sequence data, to pick up both P_{2U} and P_{2Y} receptors, if any. Only one DNA fragment was obtained in these experiments. Subsequent cloning and sequencing of this DNA fragment showed that it was 563 base-pairs in length (Figure 4-6). Overall nucleotide homology to mouse NG108-15 neuroblastoma P_{2U} was 93%, while the nucleotide similarity to chicken brain P_{2Y} was only 51%.

5'-CTCACCTGCATCAGCGTGCACCGGTGCCTGGGGGTCCTGCGCCCTCT
GCACTCCCTGAGCTGGGGCCATGCCCGCTATGCCCGCCGAGTGGCTGC
GGTTGTGTGGGTGCTAGTGCTGGCCTGCCAGGCACCCGTGCTCTACTTT
GTCACCACCAGCGTGAGAGGGACCCGAATCACCTGCCACGACACCTCG
GCCCGAGAGCTCTTTAGCCATTTTGTGGCTTACAGCTCTGTCATGCTGG
GTCTGCTTTTTGCTGTGCCCTTTTCCATCATCCTGGTCTGTTACGTGCTCA
TGGCCCGACGGCTGCTCAAACCGGCTTATGGGACCACAGGTCTGCCTC
GGGCCAAGCGCAAGTCTGTGCGCACCATCGCCTTAGTACTGGCCGTCTT
CGCCCTCTGCTTCCTGCCTTTCCACGTCACCCGCACCCTCTATTACTCCTT
CCGATCACTTGACCTCAGTTGCCACACCCTCAACGCCATCAACATGGCG
TATAAGATCACCCGGCCACTGGCCAGCGCCAACAGTTGCCTTGACCCTG
TGCTCTACTTCCTGGCAGGGCAGAG-3'

Figure 4-6. Nucleotide sequence of a cDNA fragment of ATP receptors obtained from rat pituitary.

Discussion

This study has clearly demonstrated that gonadotropes are direct targets for ATP action. This interpretation is based upon intracellular Ca^{2+} imaging experiments showing that: 1) 82% of GnRH-responsive cells also respond to ATP; 2) ATP- and GnRH-responsive pituitary cells are not responsive to the other main hypothalamic releasing hormones; and 3) nucleotide triphosphates increase cytosolic Ca^{2+} in the GnRH-responsive gonadotrope-derived αT3-1 cell line (Windle *et al.* 1990, McArdle *et al.* 1992) with a similar pharmacological profile to that observed in normal gonadotropes. This identification is further validated by the superfusion experiments which show ATP causes a significant luteinizing hormone release from pituitary cells (Chapter 5). The ATP-induced Ca^{2+}_i response in gonadotropes is specific, since the Ca^{2+}_i response is blocked by the ATP receptor antagonist suramin and among the nucleosides and nucleotides examined only ATP, ADP, UTP and UDP produce a significant response. Gonadotropes, however, are not the only target cells for ATP, since these constitute only 9% of pituitary cells in culture, whereas approximately 30% of rat pituitary cells are responsive to ATP. The identification of other ATP-responsive pituitary cells remains to be established.

The present study has characterized the subtype of ATP receptor in normal gonadotropes and αT3-1 cells by examining the agonist potency and efficacy of nucleotides and analogues. The complete absence of Ca^{2+}_i increase in cells stimulated with β,γ-methylene ATP and α,β-methylene ATP is indicative of a non-P_{2X} receptor. This is further supported by the observation that ATP-induced responses involve Ca^{2+} mobilization from intracellular pools, which is apparently distinct from the ligand-gated ion channels of P_{2X} receptors (Bean 1992, Abbracchio & Burnstock 1994). As the efficacy of 2-methylthioATP, commonly considered to be the key agonist in the definition of P_{2Y} receptors, is much less than that of ATP, and α,β-methylene ATP (a partial P_{2Y}-purinoceptor agonist) failed to produce any Ca^{2+}_i response, the receptor responsible for the Ca^{2+}_i response in gonadotropes and αT3-1 cells is distinct from the

classic P_{2Y} receptor as well. Furthermore, UTP induced a Ca^{2+}_i response with similar potency and efficacy to ATP and ADP. Thus, the ATP receptors in rat gonadotropes and αT3-1 cells exhibits the following rank-order of agonist potency and efficacy:- ATP = ADP = UTP > 2-methylthioATP >> β,γ-methylene ATP / α,β-methylene ATP. This pattern appears to fit best into the P_{2U} subtype (Dubyak 1991, Lustig *et al.* 1993) or the P_{2Y2}-purinoceptor according to the recent suggested reclassification of purinoceptors (Fredholm *et al.* 1994, Abbracchio & Burnstock 1994), and is consistent with the findings of 'nucleotide receptor' in mixed pituitary cells in culture (Davidson *et al.* 1990).

It has been reported that in some cell types, ATP receptors are heterogeneous and mediate different responses in the same biological system (Keppens 1993). Recently, two distinct receptors (P_{2Y} and P_{2U}) were found to co-exist in endothelial cells (Wilkinson *et al.* 1993, Motte *et al.* 1993), suggesting that ATP and UTP may have different binding sites in one cell type, i.e. ATP binds to both P_{2Y} and P_{2U} and UTP binds to P_{2U}. In gonadotropes and αT3-1 cells, ATP and UTP appear to be acting via common receptors because the effects of ATP and UTP together do not exceed those to either nucleotide alone (Figure 3b), as has been demonstrated in renal mesangial cells (Pfeilschifter 1990) and airway epithelial cells (Brown *et al.* 1991). This interpretation is supported by the cross-desensitization between ATP and UTP (Figure 4-5). This cross-desensitization is specific to the nucleotides in that neither pretreatment reduced subsequent responses to GnRH, and presumably reflects desensitization occurring at the level of a shared nucleotide receptor.

The molecular cloning data in this study was limited. Nevertheless, they are supportive. The primers used for RT-PCR were degenerate to both P_{2Y1} and P_{2U} purinoceptors but only one cDNA fragment was obtained. The very high similarity of this cDNA to the cloned mouse NG108-15 neuroblastoma P_{2U} (Lustig *et al.* 1993) but not to the chicken brain P_{2Y} (Webb *et al.* 1993) seems to indicate that P_{2U} rather than P_{2Y} receptors are predominantly expressed in the pituitary. The present cloning work will

be of benefit to allow further cloning of full length of cDNA encoding rat pituitary ATP receptors, as the obtained cDNA fragment is an ideal probe to screen a cDNA library. Indeed, an ATP receptor has been cloned from pituitary cells using this cDNA probe and the clone has also been expressed and functionally characterized as the P_{2U} subtype (data not presented in the thesis).

Collectively, these data indicate that the responses of gonadotropes and αT3-1 cells to these nucleotides are mediated by a single class of receptor pharmacologically characterized as the P_{2U} subtype and clearly implicate ATP receptors in the regulation of anterior pituitary function.

Chapter 5. ATP RECEPTOR-MEDIATED LUTEINIZING HORMONE RELEASE FROM PITUITARY CELLS

Introduction

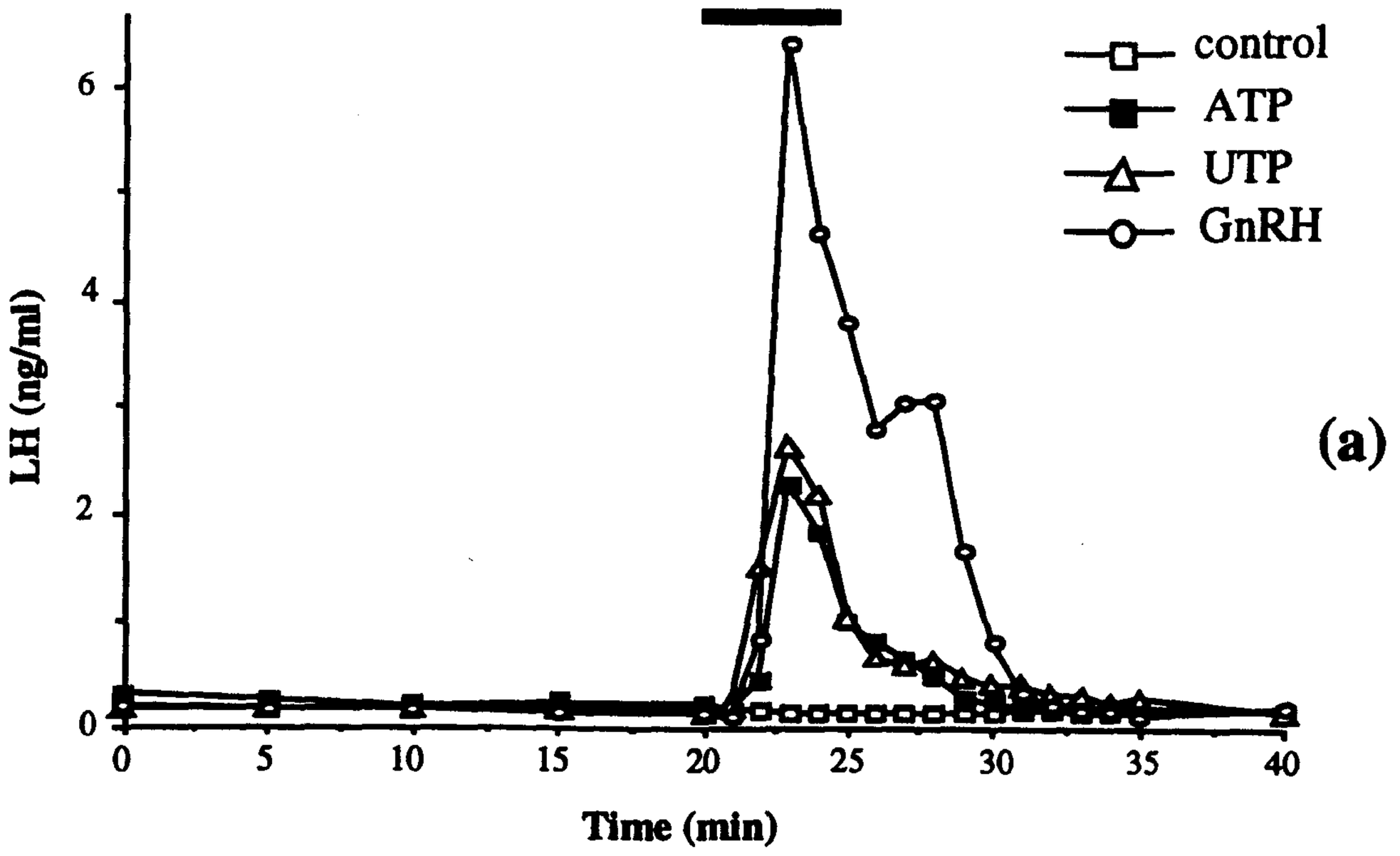
The experimental data described in the last chapter (Chapter 4) have clearly shown that ATP and UTP act on ATP receptors to provoke a rapid and dramatic increase in cytosolic Ca^{2+} in pituitary GnRH-responsive cells. Given the central importance of Ca^{2+} in the mediation of exocytotic gonadotropin release (Huckle & Conn 1988), this data suggests that the effect of P_{2U} receptor occupancy on Ca^{2+} metabolism in gonadotropes may have an important role in the regulation of their activity. To address this question, the present study employed a superfusion system to examine the effect of extracellular nucleotides on the kinetics of luteinizing hormone secretion.

Methods

Pituitary glands from 4 week-old female Wistar rats were enzymatically dispersed and after 2 days in culture, cells were subject to superfusion experiments.

Results

GnRH was a very strong stimulator of LH release (Figures 5-1a & 5-2). Addition of ATP and UTP (both 100 μM) also caused a robust (more than 14 fold) increase in the rate of LH release from a resting LH release of 125 ± 10 and 73.5 ± 7 pg/min to a maximum of 1755 ± 484 and 1261 ± 226 pg/min respectively (Mean ± SE: n = 3) (Figure 5-1a). Repetitive stimulation with ATP and UTP (both 6 μM) produced corresponding repetitive increases in LH release (Figure 5-1b). The LH release mediated by nucleotides was concentration-dependent (Figure 5-2). Near maximal



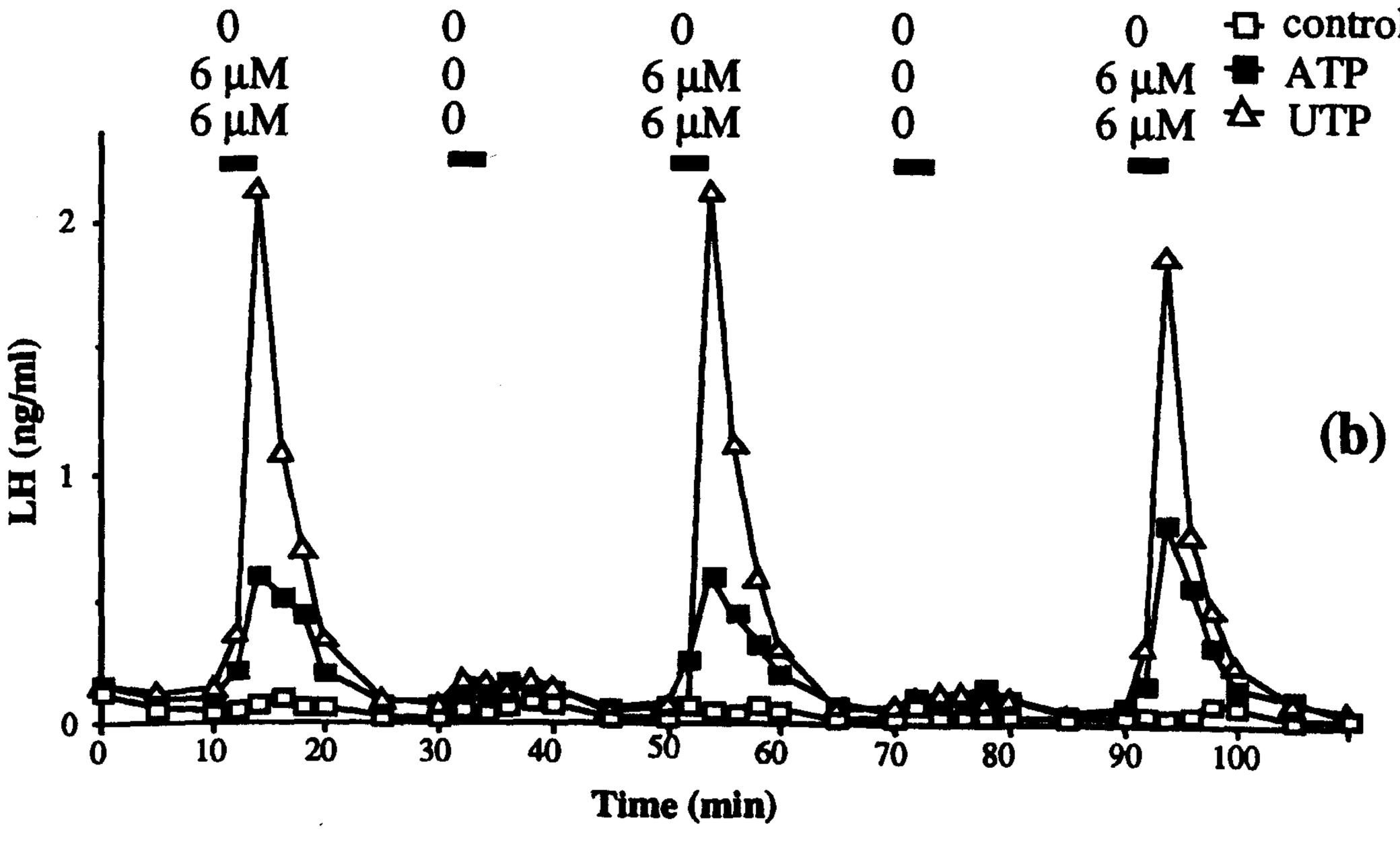


Figure 5-1. ATP and UTP-induced LH release from superfused rat pituitary cells. (a) Representative examples showing stimulation with ATP (100 µM), UTP (100 µM), GnRH (10 nM) or vehicle alone for 5 min (indicated by the bar). (b) The effects of repetitive stimulation with ATP (6 µM), UTP (6 µM) or vehicle, each for 4 min (indicated by the bars). Each trace is representative recording from 3 - 4 separate experiments.

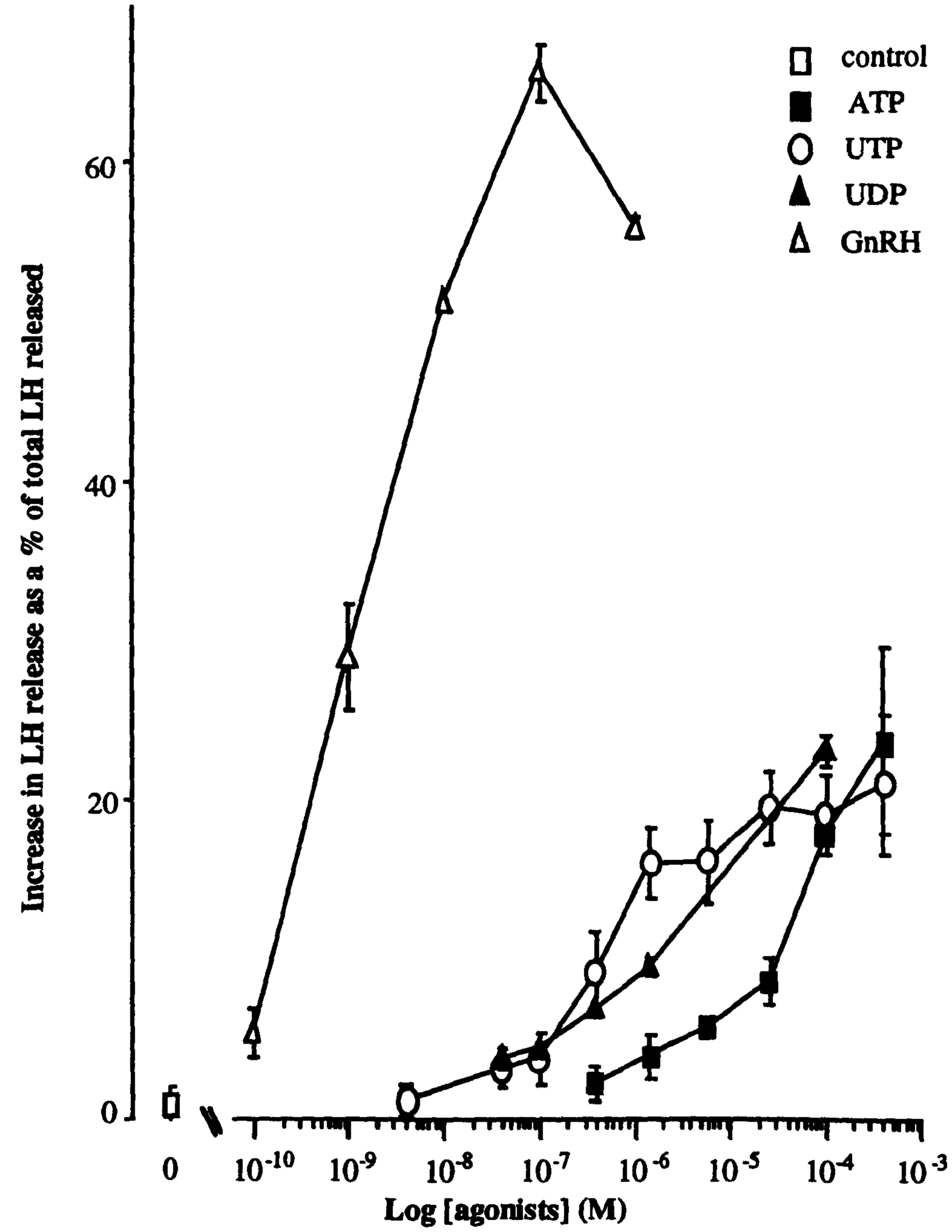


Figure 5-2. Concentration-dependent stimulation of LH release. Superfused rat pituitary cells were stimulated with ligand (for 5 min), followed, 30 mins later, by a standard dose of the calcium ionophore A23187 (5 μM for 5 mins). The results shown indicate the total LH released during the 10 min following ligand addition, as a percentage of the total LH released within the experimental period of 60 min (including the basal LH release during the 20 min before addition of the ligand, the release in response to both ligand and A23187 and the release between the two). The results were thus corrected for potential depletion of the releasable LH pool and for overall response differences between experiments. Each data point (mean ± SE) was derived from 2 experiments for GnRH and UDP and from 3 experiments for ATP and UTP. In the control group (n = 5), the "agonist" was either superfusion medium or thyrotropin-releasing hormone (0.1 or 1 nM).

effect was obtained with 1.5 μM UTP or 100 μM ATP and the EC50 value for UTP and ATP action was approximately 0.5 μM and 40 μM, respectively. The maximal effect of both ATP and UTP was similar, amounting to about 30% of the maximal GnRH response. In the Ca^{2+}-free medium containing 50 μM EGTA, the maximal UTP (6 μM)-induced LH release rate was attenuated by approximately 25%.

Addition of UDP (100 μM) caused a significant increase in LH release from superfused cells as well (Figure 5-3c). The EC50 value for UDP was approximately 3 μM with a maximal effect comparable to both UTP and ATP (Figure 5-2).

Among other nucleosides and nucleotides examined, adenosine, AMP, CDP, CTP and UMP (all 100 μM) had no effect on LH release from the superfused pituitary cells, while ADP, GDP and GTP (all 100 μM) had only modest stimulatory effects (Figure 5-3). The P_{2X} receptor agonists β,γ-methylene ATP and α,β-methylene ATP (both 100 μM) did not stimulate LH release, and 2-methylthioATP (P_{2Y} receptor agonist) had only a modest stimulatory effect (Figure 5-3d).

The simultaneous application of UTP and ATP (both 100 μM) to superfused rat pituitary cells, failed to elicit a greater LH release than that obtained with either nucleotides alone (Figure 5-4a). Pretreatment with 100 μM UTP for 5 min considerably reduced the subsequent LH release in response to UTP or ATP (both 100 μM) (Figure 5-4b & 4c).

Discussion

In the mouse gonadotrope-derived αT3-1 cell line, ATP, ADP and UTP were found to be equipotent in eliciting an intracellular Ca^{2+} response (Chen *et al.* 1994b), while in superfused rat pituitary cells in primary culture, the following rank-order of potency in stimulation of LH release was observed: UTP > ATP > ADP. The maximal effect of

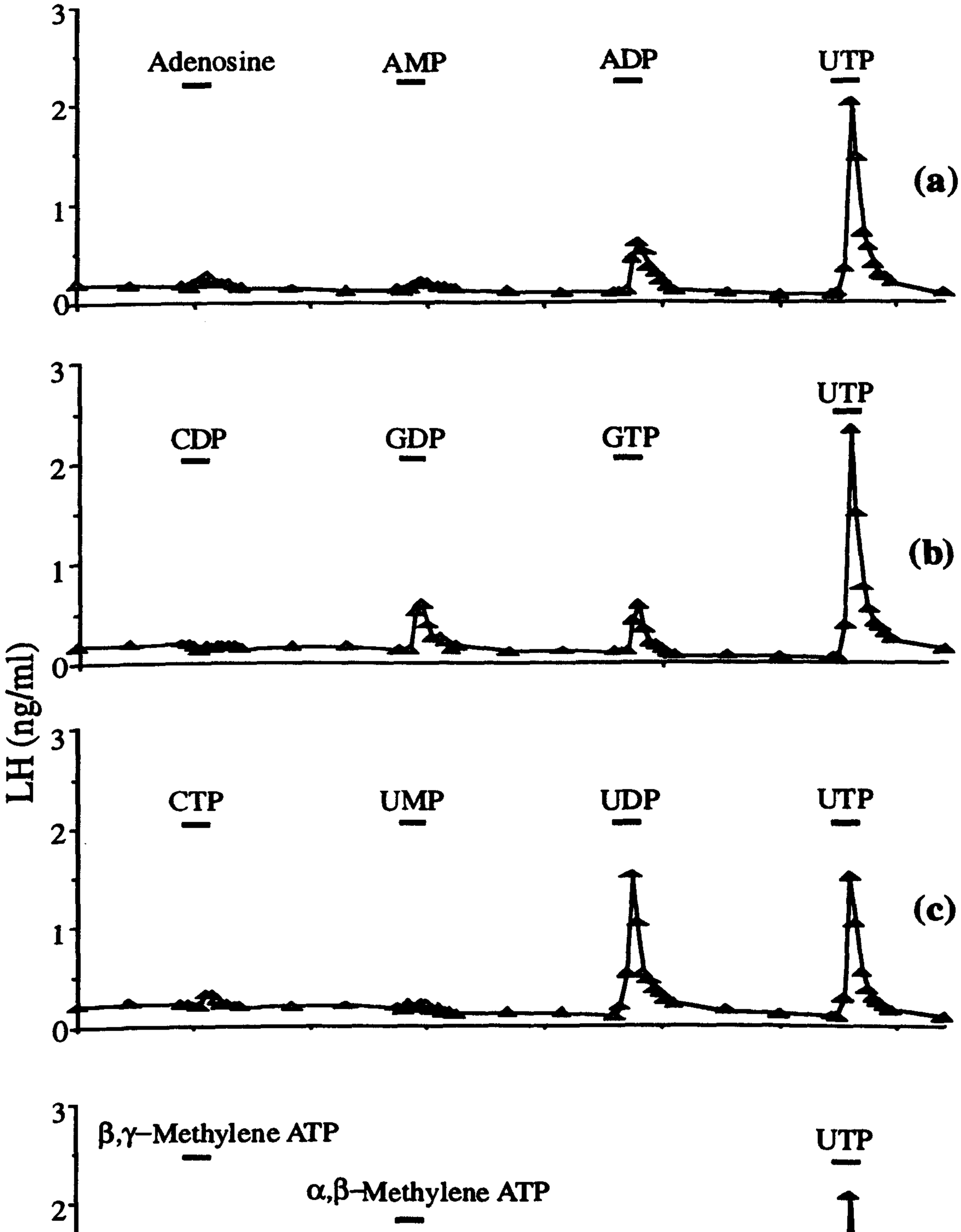


Figure 5-3. Effects of nucleoside and nucleotides on LH release. Cells were stimulated as indicated with various compounds (all 100 μM) for 5 min. Each panel shows representative recording from 2 separate experiments. Superfusate fractions were collected for periods of 1 or 9 min.

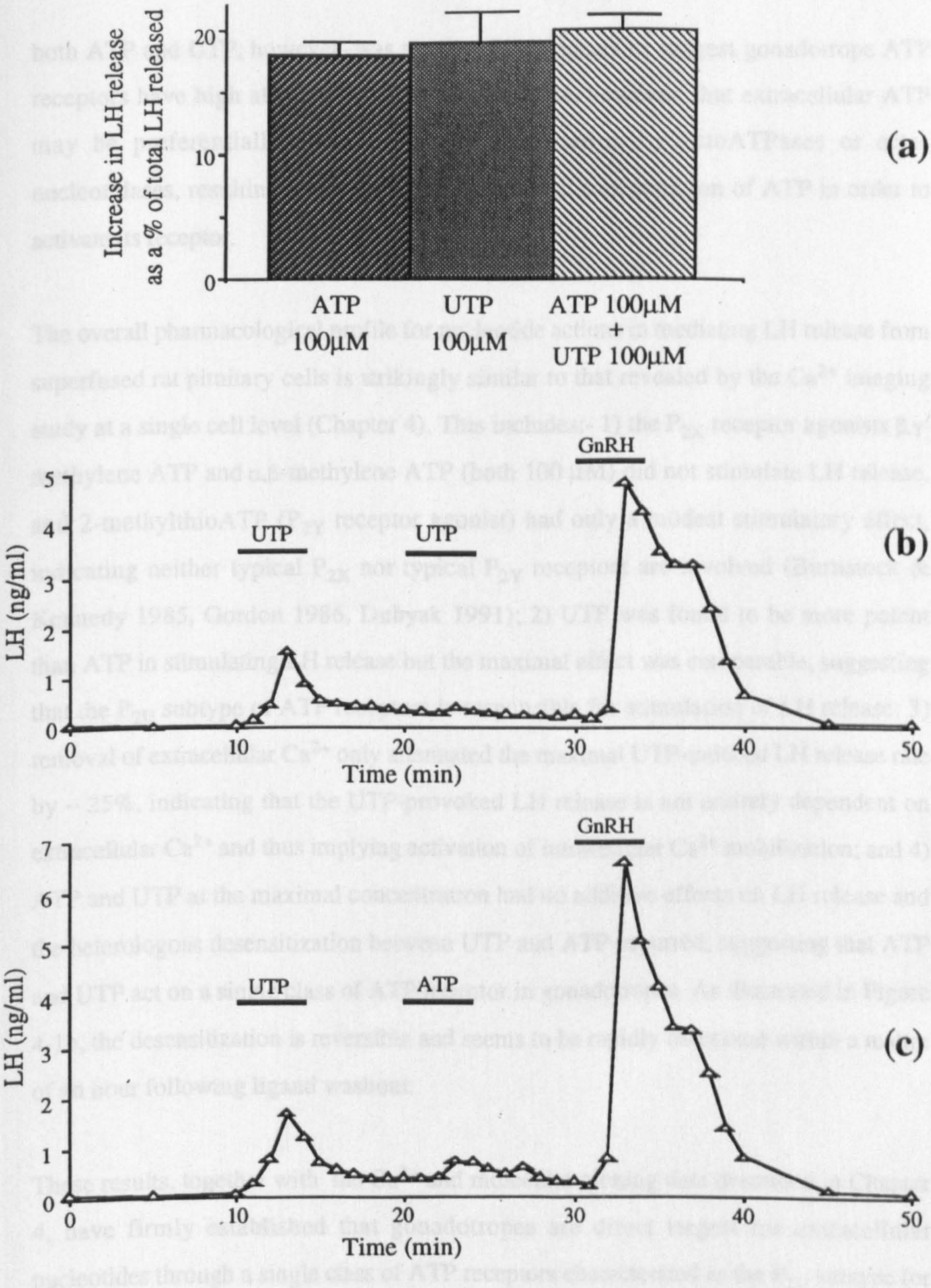


Figure 5-4. Effects of ATP and UTP on LH release. (a) Cells were stimulated with ATP and UTP alone or in combination as indicated. Experiments and data analysis were performed as described in the legend of Figure 2. The figure shows the mean ± SE (n = 3: no significant differences between groups using an unpaired t-test). (b & c) Homologous and heterologous desensitization of LH release. Cells were stimulated as indicated with UTP, ATP (both 100 μM) and GnRH (10 nM). Panels b &c show representative recording from 2 separate experiments.

both ATP and UTP, however, was similar. This appears to suggest gonadotrope ATP receptors have high affinity for UTP. It is possible, however, that extracellular ATP may be preferentially subject to rapid degradation by ectoATPases or ecto-nucleotidases, resulting in a requirement for a high concentration of ATP in order to activate its receptor.

The overall pharmacological profile for nucleotide actions in mediating LH release from superfused rat pituitary cells is strikingly similar to that revealed by the Ca^{2+} imaging study at a single cell level (Chapter 4). This includes:- 1) the P_{2X} receptor agonists β,γ-methylene ATP and α,β-methylene ATP (both 100 μM) did not stimulate LH release, and 2-methylthioATP (P_{2Y} receptor agonist) had only a modest stimulatory effect, indicating neither typical P_{2X} nor typical P_{2Y} receptors are involved (Burnstock & Kennedy 1985, Gordon 1986, Dubyak 1991); 2) UTP was found to be more potent than ATP in stimulating LH release but the maximal effect was comparable, suggesting that the P_{2U} subtype of ATP receptors is responsible for stimulation of LH release; 3) removal of extracellular Ca^{2+} only attenuated the maximal UTP-induced LH release rate by ~ 25%, indicating that the UTP-provoked LH release is not entirely dependent on extracellular Ca^{2+} and thus implying activation of intracellular Ca^{2+} mobilization; and 4) ATP and UTP at the maximal concentration had no additive effects on LH release and the heterologous desensitization between UTP and ATP occurred, suggesting that ATP and UTP act on a single class of ATP receptor in gonadotropes. As illustrated in Figure 4-1b, the desensitization is reversible and seems to be rapidly overcome within a matter of an hour following ligand washout.

These results, together with the Ca^{2+} and molecular cloning data described in Chapter 4, have firmly established that gonadotropes are direct targets for extracellular nucleotides through a single class of ATP receptors characterized as the P_{2U} subtype (or P_{2Y2} subtype as recently suggested (Abbracchio & Burnstock 1994)). Activation of these receptors leads to a significant increase in the rate of gonadotropin release. This data further reinforces the idea that the pituitary ATP receptors play an important -

presumably modulatory - role in the regulation of pituitary gonadotrope.

Chapter 6. ATP RECEPTOR-MEDIATED INTRACELLULAR RESPONSES IN GONADOTROPE-DERIVED αT3-1 CELLS

Introduction

ATP and other nucleotides act on a large and diverse family of P_2 purinoceptors (Burnstock 1986, Gordon 1986), four of which have recently been cloned (Lustig *et al.* 1993, Webb *et al.* 1993, Brake *et al.* 1994, Valera *et al.* 1994). This receptor family can be divided into two distinct classes, the first being ligand-gated ion channels (P_{2X} receptors) and the second consisting of G protein-coupled receptors (P_{2Y}, P_{2U}, P_{2T} and P_{2D}). Activation of the latter receptor group has been shown to lead to a complex signal transduction cascade involving inositol phosphate accumulation, Ca^{2+} mobilization, changes in cAMP production and activation of phospholipase A2.

The data from this study have demonstrated that ATP increases intracellular Ca^{2+} concentration in a subpopulation of rat pituitary cells and we have identified gonadotropes as one of the target cells for ATP action via ATP receptors of the P_{2U} subtype. Activation of this receptor causes a significant release of luteinizing hormone from superfused rat pituitary cells. In order to elucidate the possible signal transduction mechanisms subserving ATP actions in gonadotropes, the present study was to examine intracellular events other than inositol phosphate accumulation (which has already been well studied in pituitary cells (Davidson *et al.* 1990)) in response to ATP in a gonadotrope-derived αT3-1 cells bearing P_{2U} receptors similar to those found on gonadotropes (Chen *et al.* 1994c).

Methods

αT3-1 cells were cultured in 24-well culture plates for cAMP and cGMP assays or in 75 cm^2 culture flasks for Western immunoblotting of protein kinase C. For calcium ion

imaging at the single cell level, αT3-1 cells were harvested by trypsinization and then incubated for 2 days on 22 mm diameter glass coverslips. Cells were exposed to ligand by rapidly pipetting 2.5 ml of medium containing stimuli into the recording chamber which was maintained at approximately 0.5 ml by means of an aspiration tube positioned 2 mm above the coverslip. Cells with a clear intracellular Ca^{2+} response (i.e. those in which stimulation at least doubled the $[Ca^{2+}]_i$) were defined as 'ligand-responsive' and used to generate the data shown. In the present experimental condition, approximately 65% of αT3-1 cells were found to be ATP- or UTP (both 100 μM)-responsive and more than 90% of the cells were GnRH (10 nM)-sensitive. Ca^{2+}-free solution was made by omitting $CaCl_2$ and adding 0.05 mM EGTA to Ca^{2+} buffer buffer. All experiments were repeated on at least three occasions and data shown are either from a single representative experiment or are pooled from multiple experiments as described. Means ± SE are given throughout. Paired or unpaired *t*-tests were performed where appropriate.

Results

Intracellular Ca^{2+} signalling

The addition of 100 μM ATP to fura-2 loaded αT3-1 cells produced a biphasic cytosolic Ca^{2+} response: a transient increase (spike) in $[Ca^{2+}]_i$ from a resting $[Ca^{2+}]_i$ of 36 ± 0.4 nM to a maximum $[Ca^{2+}]_i$ of 297 ± 27 nM (Mean ± SE: n = 26 cells of 3 separate experiments) followed by a small plateau lasting for about 4 minutes (Figure 6-1). The basal level of $[Ca^{2+}]_i$ in αT3-1 cells was similar to that reported by others who used the similar methodology (McArdle *et al.* 1992, Anderson *et al.* 1992). The intracellular Ca^{2+} response was relatively rapid and reached the peak between 4 - 17 sec (mean = 8 sec: n = 26 cells) after addition of ATP. Equimolar concentrations of UTP induced a biphasic Ca^{2+} response similar to that induced by ATP.

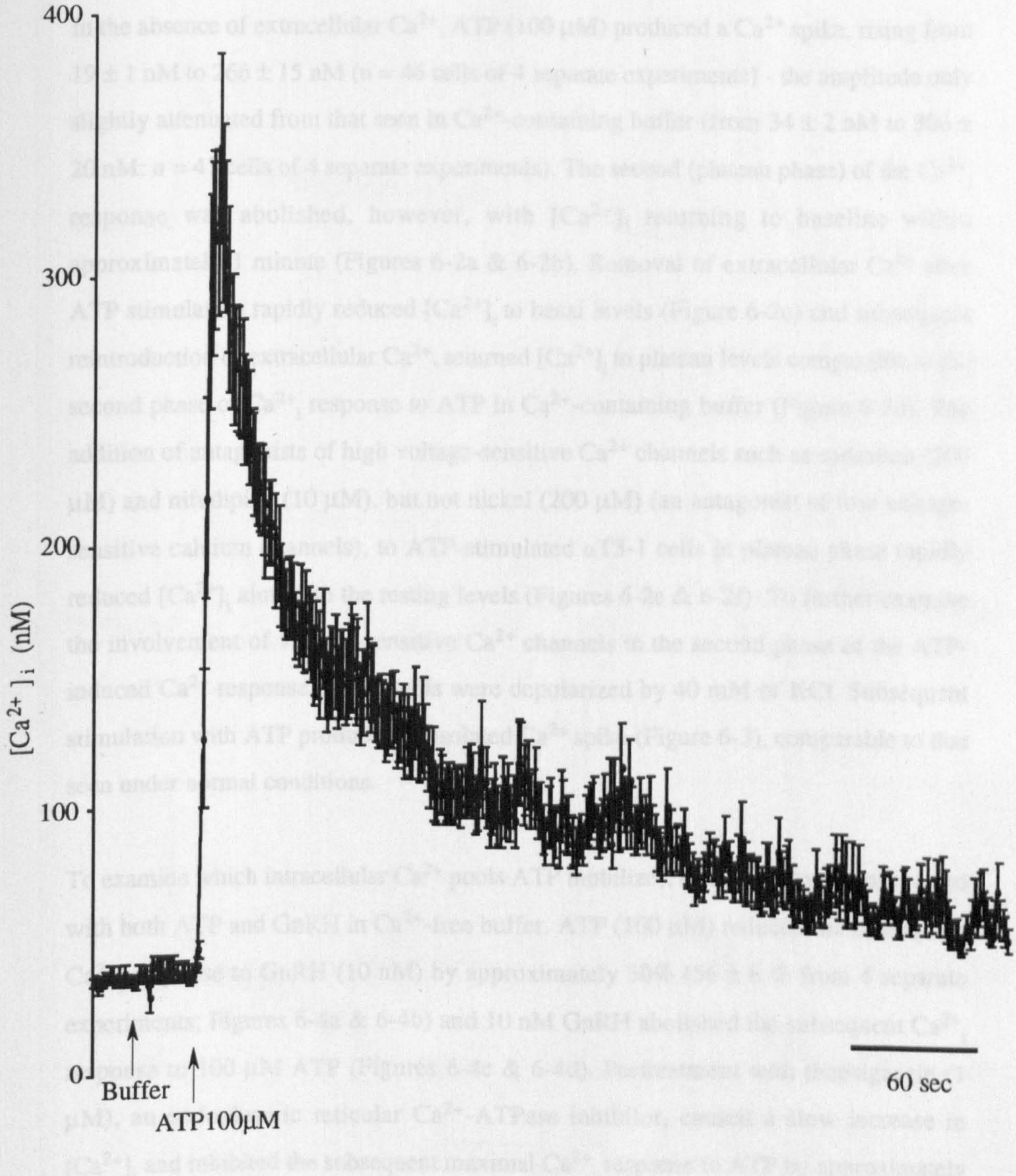


Figure 6-1. Intracellular Ca^{2+} increase in response to ATP in αT3-1 cells. The trace shown is pooled from 9 single cells in one representative experiment (mean ± SE). Addition of buffer alone or buffer containing ligand at the final concentration is indicated by the arrow.

In the absence of extracellular Ca^{2+}, ATP (100 μM) produced a Ca^{2+} spike, rising from 19 ± 1 nM to 266 ± 15 nM (n = 46 cells of 4 separate experiments) - the amplitude only slightly attenuated from that seen in Ca^{2+}-containing buffer (from 34 ± 2 nM to 306 ± 20 nM: n = 41 cells of 4 separate experiments). The second (plateau phase) of the Ca^{2+}_i response was abolished, however, with $[Ca^{2+}]_i$ returning to baseline within approximately 1 minute (Figures 6-2a & 6-2b). Removal of extracellular Ca^{2+} after ATP stimulation rapidly reduced $[Ca^{2+}]_i$ to basal levels (Figure 6-2c) and subsequent reintroduction of extracellular Ca^{2+}, returned $[Ca^{2+}]_i$ to plateau levels comparable to the second phase of Ca^{2+}_i response to ATP in Ca^{2+}-containing buffer (Figure 6-2d). The addition of antagonists of high voltage-sensitive Ca^{2+} channels such as cadmium (200 μM) and nifedipine (10 μM), but not nickel (200 μM) (an antagonist of low voltage-sensitive calcium channels), to ATP-stimulated αT3-1 cells in plateau phase rapidly reduced $[Ca^{2+}]_i$ almost to the resting levels (Figures 6-2e & 6-2f). To further examine the involvement of voltage-sensitive Ca^{2+} channels in the second phase of the ATP-induced Ca^{2+} response, αT3-1 cells were depolarized by 40 mM of KCl. Subsequent stimulation with ATP produced an isolated Ca^{2+} spike (Figure 6-3), comparable to that seen under normal conditions.

To examine which intracellular Ca^{2+} pools ATP mobilizes, αT3-1 cells were stimulated with both ATP and GnRH in Ca^{2+}-free buffer. ATP (100 μM) reduced the subsequent Ca^{2+}_i response to GnRH (10 nM) by approximately 50% (56 ± 8 % from 4 separate experiments; Figures 6-4a & 6-4b) and 10 nM GnRH abolished the subsequent Ca^{2+}_i response to 100 μM ATP (Figures 6-4c & 6-4d). Pretreatment with thapsigargin (1 μM), an endoplasmic reticular Ca^{2+}-ATPase inhibitor, caused a slow increase in $[Ca^{2+}]_i$ and inhibited the subsequent maximal Ca^{2+}_i response to ATP by approximately 60% (63 ± 4 % from 3 separate experiments; Figure 6-5). Pretreatment with ryanodine (1 - 50 μM for 5 min), a blocker of intracellular Ca^{2+} release in some types of cell, however, had no effect on either basal levels of $[Ca^{2+}]_i$ or ATP- and GnRH-induced Ca^{2+}_i responses in αT3-1 cells.

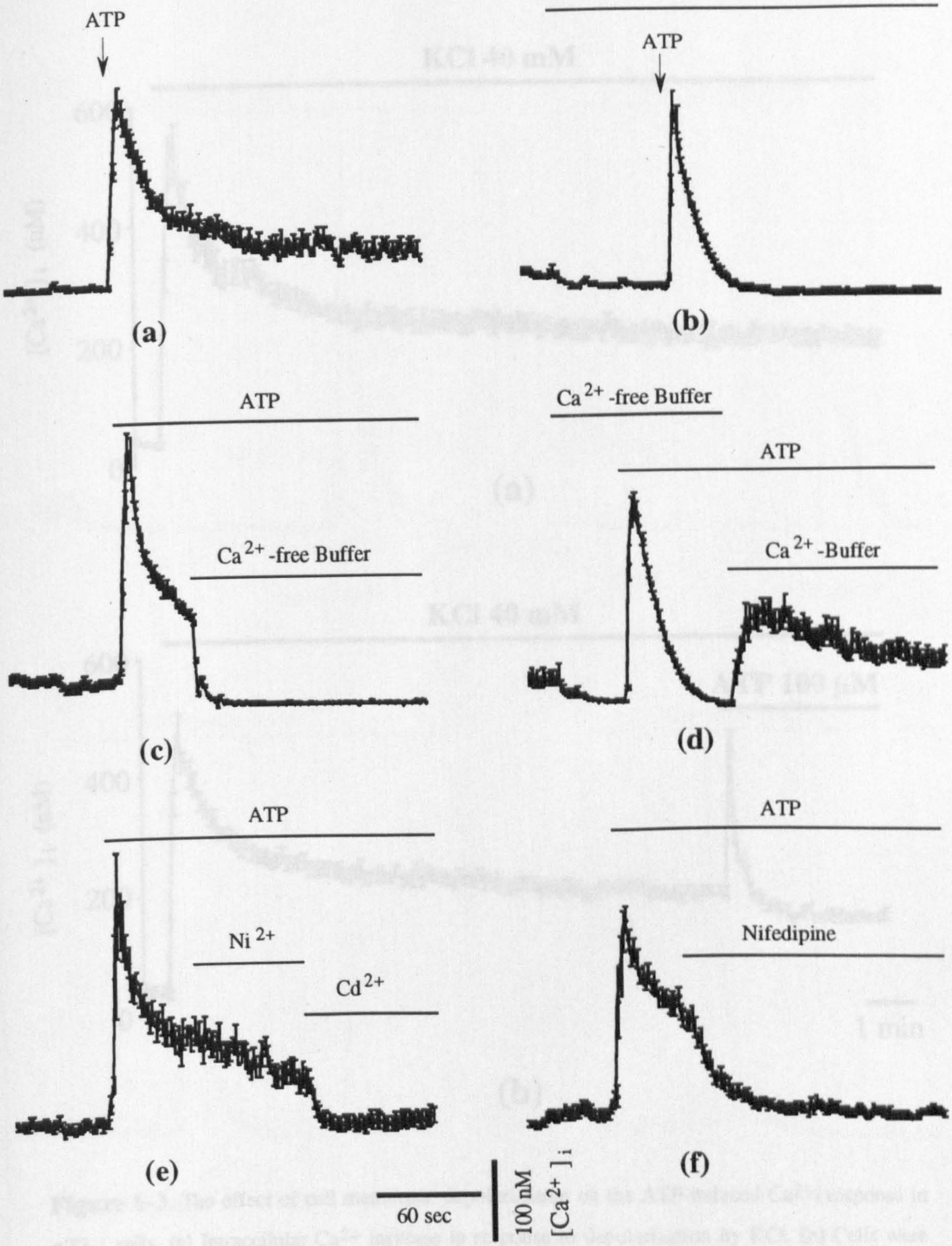


Figure 6-2. The effect of extracellular Ca^{2+} on the ATP-induced $Ca^{2+}i$ increase in αT3-1 cells. (a & b) The cells were stimulated with ATP in Ca^{2+}-containing buffer (a) and in Ca^{2+}-free buffer containing 0.05 mM EGTA (b). (c) Ca^{2+}-containing buffer was exchanged for Ca^{2+}-free buffer after αT3-1 cells had been stimulated with ATP. (d) Ca^{2+}-free buffer was exchanged for Ca^{2+}-containing buffer after αT3-1 cells had been stimulated with ATP. (e & f) 200 μM of Ni^{2+} or Cd^{2+} and 10 μM of nifedipine was introduced after αT3-1 cells had been stimulated with ATP. Each trace is pooled from ≥ 7 single cells in one separate representative experiment (mean ± SE). Buffer changes and ligand additions (such as 100 μM ATP) are indicated either by the arrow or the bar.

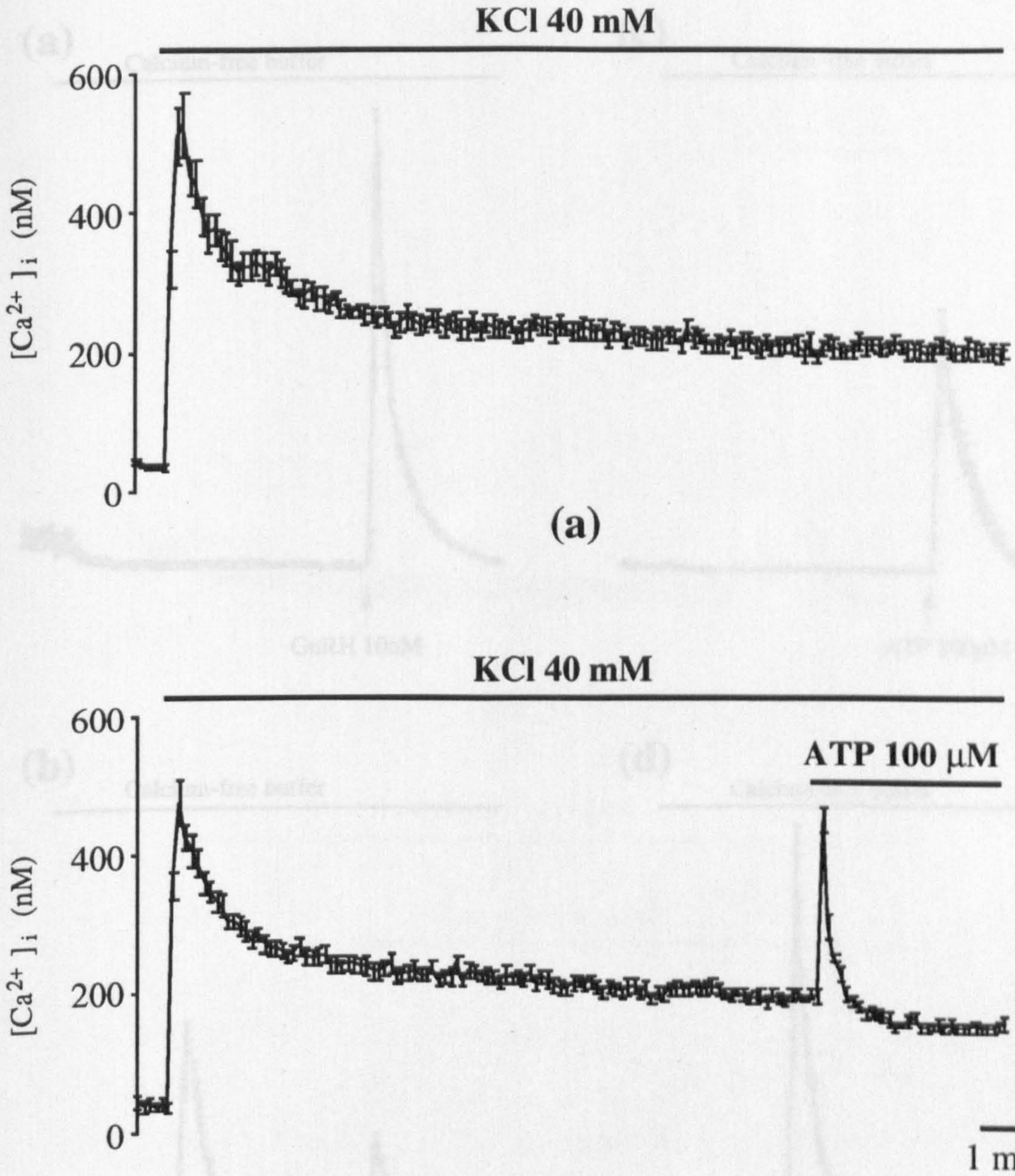


Figure 6-3. The effect of cell membrane depolarization on the ATP-induced $Ca^{2+}i$ response in αT3-1 cells. (a) Intracellular Ca^{2+} increase in response to depolarization by KCl. (b) Cells were depolarized with KCl and subsequently challenged with ATP. Each trace is pooled from ≥ 5 single cells in one separate representative experiment (mean ± SE) and applications of agents are indicated by the bars.

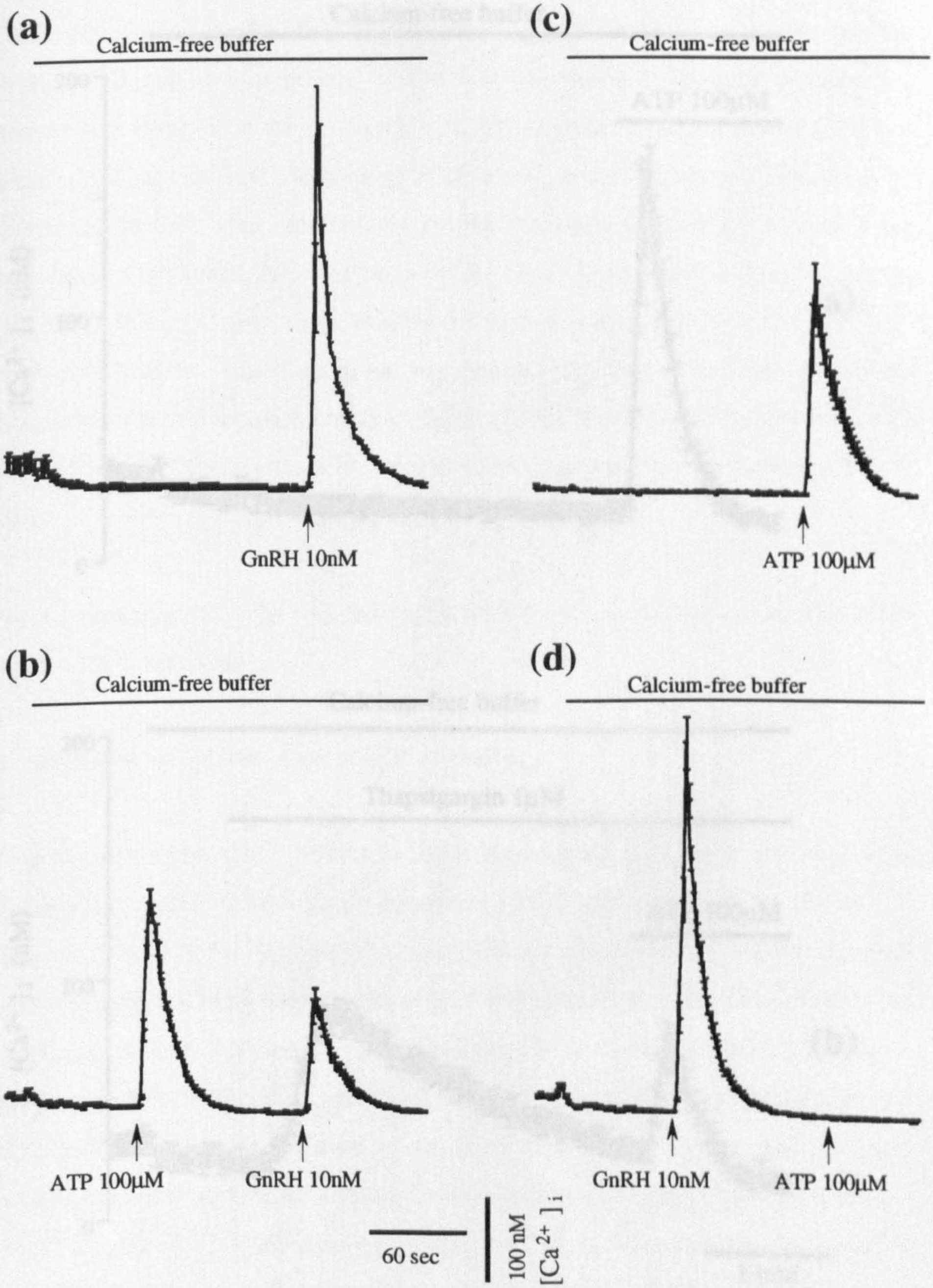


Figure 6-4. Extracellular ATP releases Ca^{2+} from GnRH-sensitive Ca^{2+} pools in αT3-1 cells. Each trace is pooled from ≥ 6 single cells in one separate representative experiment (mean ± SE). The applications of Ca^{2+}-free buffer containing 0.05 mM EGTA and ligands are indicated by the bar and the arrow.

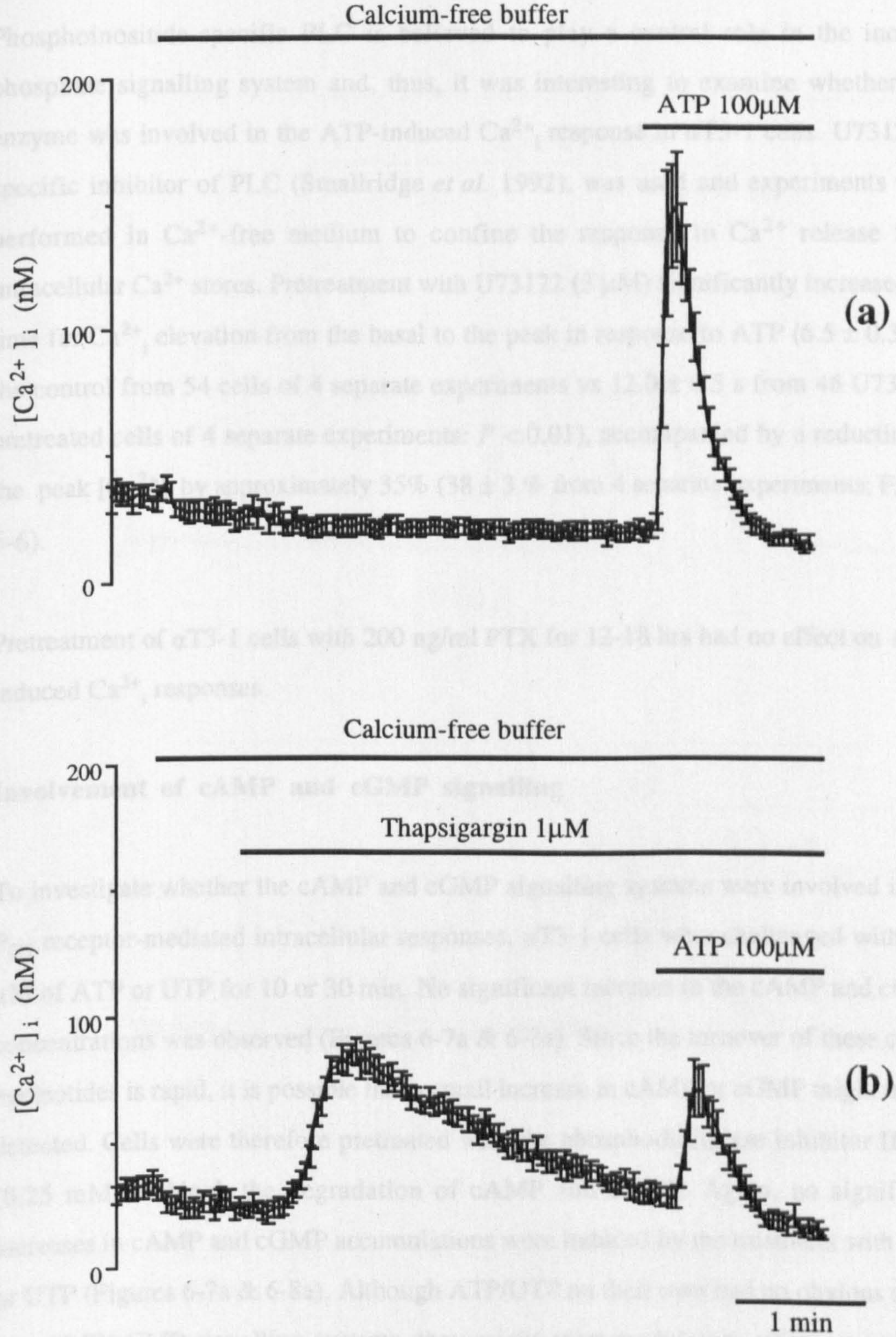


Figure 6-5. Extracellular ATP mobilizes Ca^{2+} from thapsigargin-sensitive Ca^{2+} pools in αT3-1 cells. Each trace is pooled from ≥ 8 single cells in one separate representative experiment (mean ± SE). The applications of Ca^{2+}-free buffer containing 0.05 mM EGTA and ligands are indicated by the bar and the arrow.

Phosphoinositide-specific PLC is believed to play a central role in the inositol phosphate signalling system and, thus, it was interesting to examine whether this enzyme was involved in the ATP-induced Ca^{2+}_i response in αT3-1 cells. U73122, a specific inhibitor of PLC (Smallridge *et al.* 1992), was used and experiments were performed in Ca^{2+}-free medium to confine the response to Ca^{2+} release from intracellular Ca^{2+} stores. Pretreatment with U73122 (3 μM) significantly increased the time for Ca^{2+}_i elevation from the basal to the peak in response to ATP (6.5 ± 0.3 s of the control from 54 cells of 4 separate experiments vs 12.0 ± 0.5 s from 46 U73122-pretreated cells of 4 separate experiments: $P < 0.01$), accompanied by a reduction of the peak $[Ca^{2+}]_i$ by approximately 35% (38 ± 3 % from 4 separate experiments; Figure 6-6).

Pretreatment of αT3-1 cells with 200 ng/ml PTX for 12-18 hrs had no effect on ATP-induced Ca^{2+}_i responses.

Involvement of cAMP and cGMP signalling

To investigate whether the cAMP and cGMP signalling systems were involved in the P_{2U} receptor-mediated intracellular responses, αT3-1 cells were challenged with 100 μM of ATP or UTP for 10 or 30 min. No significant increase in the cAMP and cGMP concentrations was observed (Figures 6-7a & 6-8a). Since the turnover of these cyclic nucleotides is rapid, it is possible that a small increase in cAMP or cGMP might not be detected. Cells were therefore pretreated with the phosphodiesterase inhibitor IBMX (0.25 mM) to block the degradation of cAMP and cGMP. Again, no significant increases in cAMP and cGMP accumulations were induced by the treatment with ATP or UTP (Figures 6-7a & 6-8a). Although ATP/UTP on their own had no obvious effect on cAMP/cGMP signalling systems, they might exert modulatory effect on agonist-stimulated cAMP/cGMP responses. To assess this possibility, αT3-1 cells were stimulated with forskolin, pituitary adenylate cyclase-activating polypeptide 38 (PACAP38) (McArdle *et al.* 1994) and C-type natriuretic peptide (CNP) (McArdle *et*

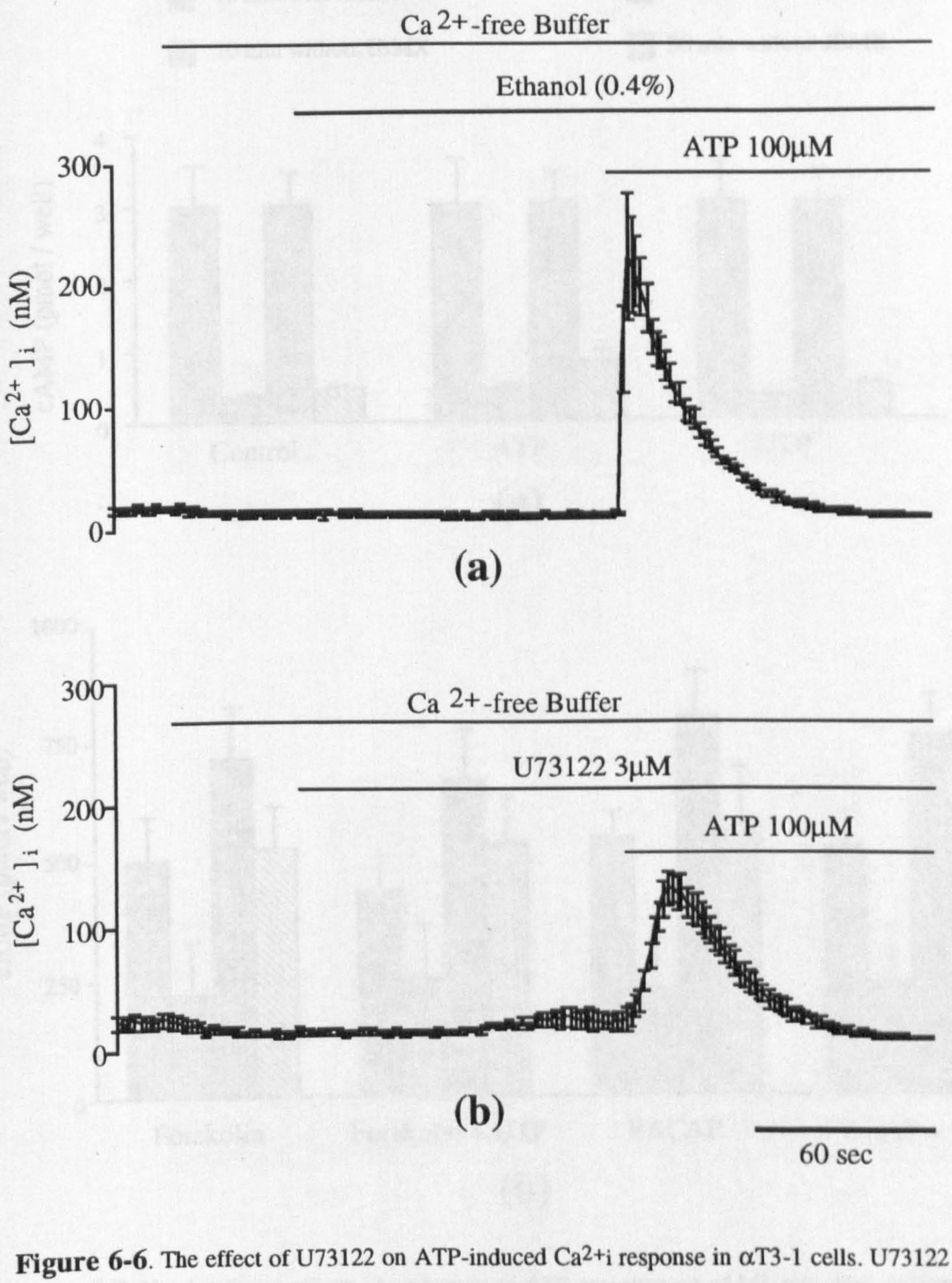


Figure 6-6. The effect of U73122 on ATP-induced $Ca^{2+}i$ response in αT3-1 cells. U73122 was dissolved in ethanol and diluted in Ca^{2+}-free buffer and the final concentration of ethanol was 0.4%, which had no apparent effect on $[Ca^{2+}]i$ as shown in the trace (a). Each trace is pooled from ≥ 7 single cells in one separate representative experiment (mean ± SE), and applications of Ca^{2+}-free buffer containing 0.05 mM EGTA and ligands are indicated by the bar and the arrow.

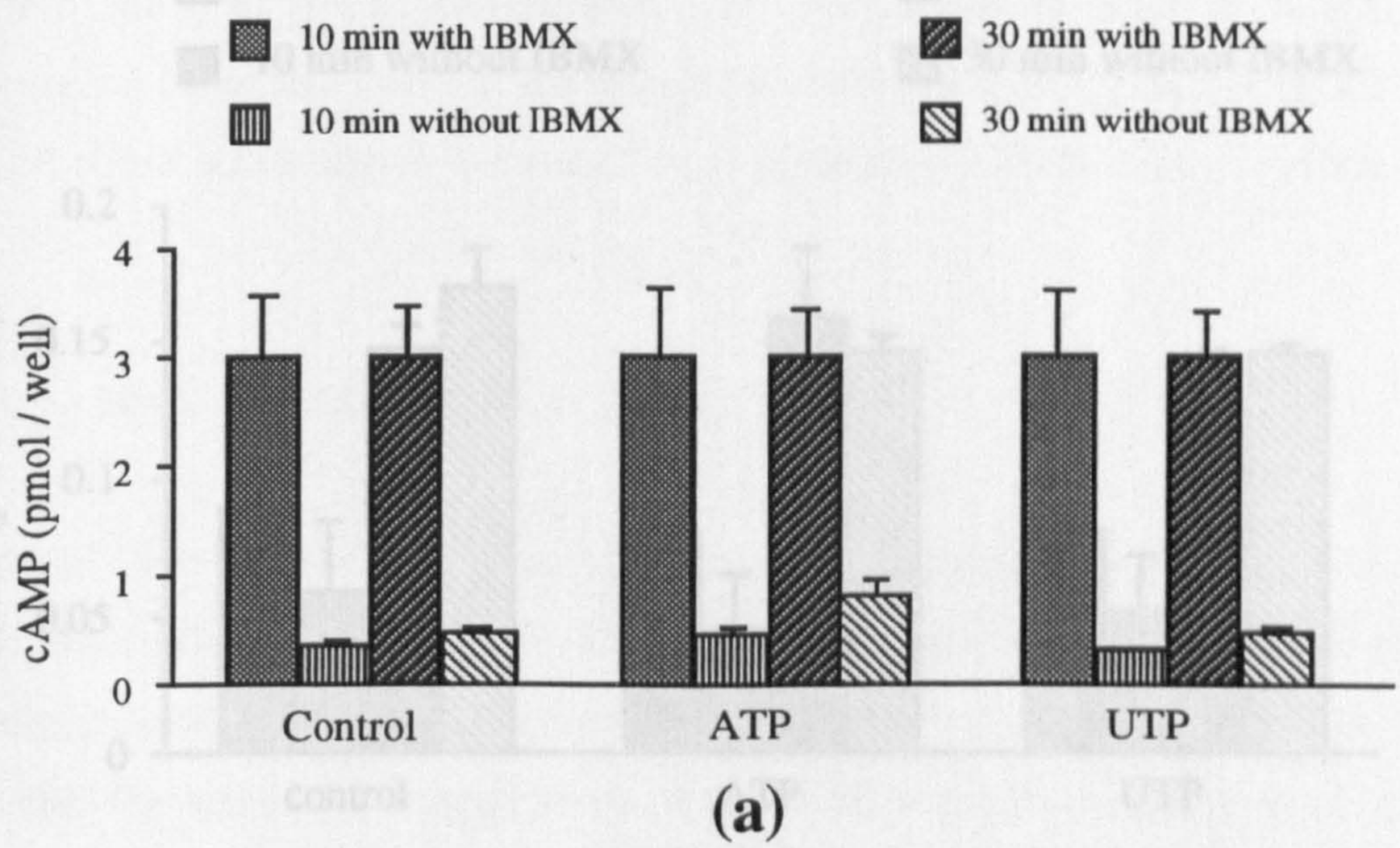


Figure 6-7. No significant effects of activation of ATP receptors on cAMP signalling systems in αT3-1 cells. Cells were preincubated in 0.25 ml of BSS with 0 or 0.25 mM 3-isobutyl-1-methylxanthine (IBMX) at 37°C for 10 min, and stimulated with ATP (100 μM), UTP (100 μM), forskolin (2 μM) and PACAP38 (10 nM) alone or in combination as indicated for 10 or 30 min in the absence or presence of IBMX. Values are mean ± SE of 3 independent experiments in triplicate. Paired t-test was used and no significant difference ($P > 0.05$) was found between the relevant groups.

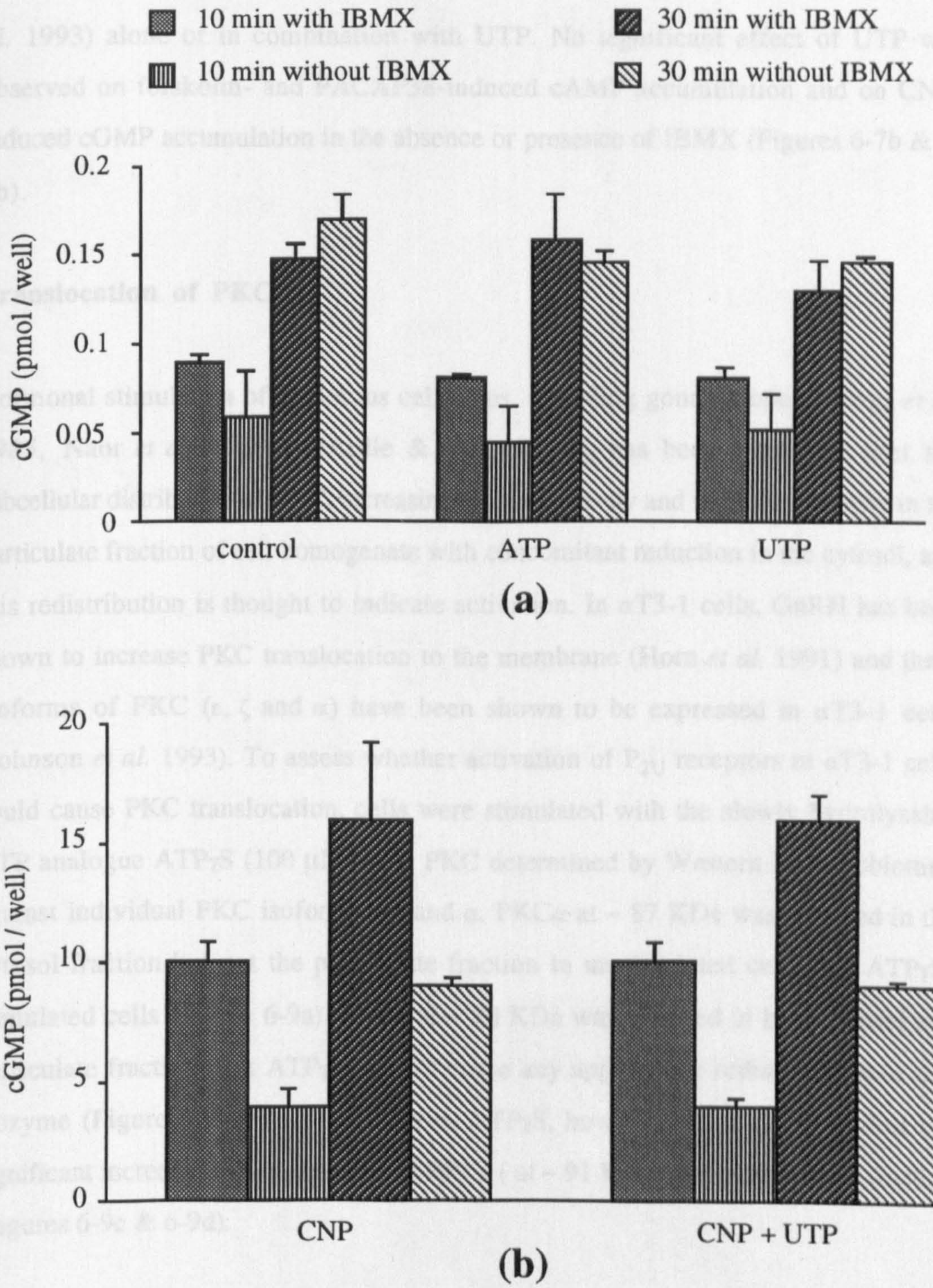


Figure 6-8. No significant effects of activation of ATP receptors on cGMP signalling systems in αT3-1 cells. Cells were preincubated in 0.25 ml of BSS with 0 or 0.25 mM 3-isobutyl-1-methylxanthine (IBMX) at 37°C for 10 min, and stimulated with ATP (100 μM), UTP (100 μM) and CNP (10 nM) alone or in combination as indicated for 10 or 30 min in the absence or presence of IBMX. Values are mean ± SE of 3 independent experiments in triplicate. Paired t-test was used and no significant difference ($P > 0.05$) was found between the relevant groups.

al. 1993) alone or in combination with UTP. No significant effect of UTP was observed on forskolin- and PACAP38-induced cAMP accumulation and on CNP-induced cGMP accumulation in the absence or presence of IBMX (Figures 6-7b & 6-8b).

Translocation of PKC

Hormonal stimulation of numerous cell types, including gonadotropes (Hirota *et al.* 1985, Naor *et al.* 1985, McArdle & Conn 1986), has been shown to alter the subcellular distribution of PKC, increasing enzyme activity and immunoreactivity in the particulate fraction of cell homogenate with concomitant reduction in the cytosol, and this redistribution is thought to indicate activation. In αT3-1 cells, GnRH has been shown to increase PKC translocation to the membrane (Horn *et al.* 1991) and three isoforms of PKC (ε, ζ and α) have been shown to be expressed in αT3-1 cells (Johnson *et al.* 1993). To assess whether activation of P_{2U} receptors in αT3-1 cells could cause PKC translocation, cells were stimulated with the slowly hydrolysable ATP analogue ATPγS (100 μM) and PKC determined by Western immunoblotting against individual PKC isoforms ε, ζ and α. PKCα at ~ 87 KDa was detected in the cytosol fraction but not the particulate fraction in unstimulated cells and ATPγS-stimulated cells (Figure 6-9a). PKCζ at ~ 80 KDa was detected in both cytosol and particulate fractions but ATPγS did not cause any appreciable redistribution of this isozyme (Figure 6-9b). Stimulation with ATPγS, however, produced a modest but significant increase in the proportion of PKCε (at ~ 91 KDa) in the particulate fraction (Figures 6-9c & 6-9d).

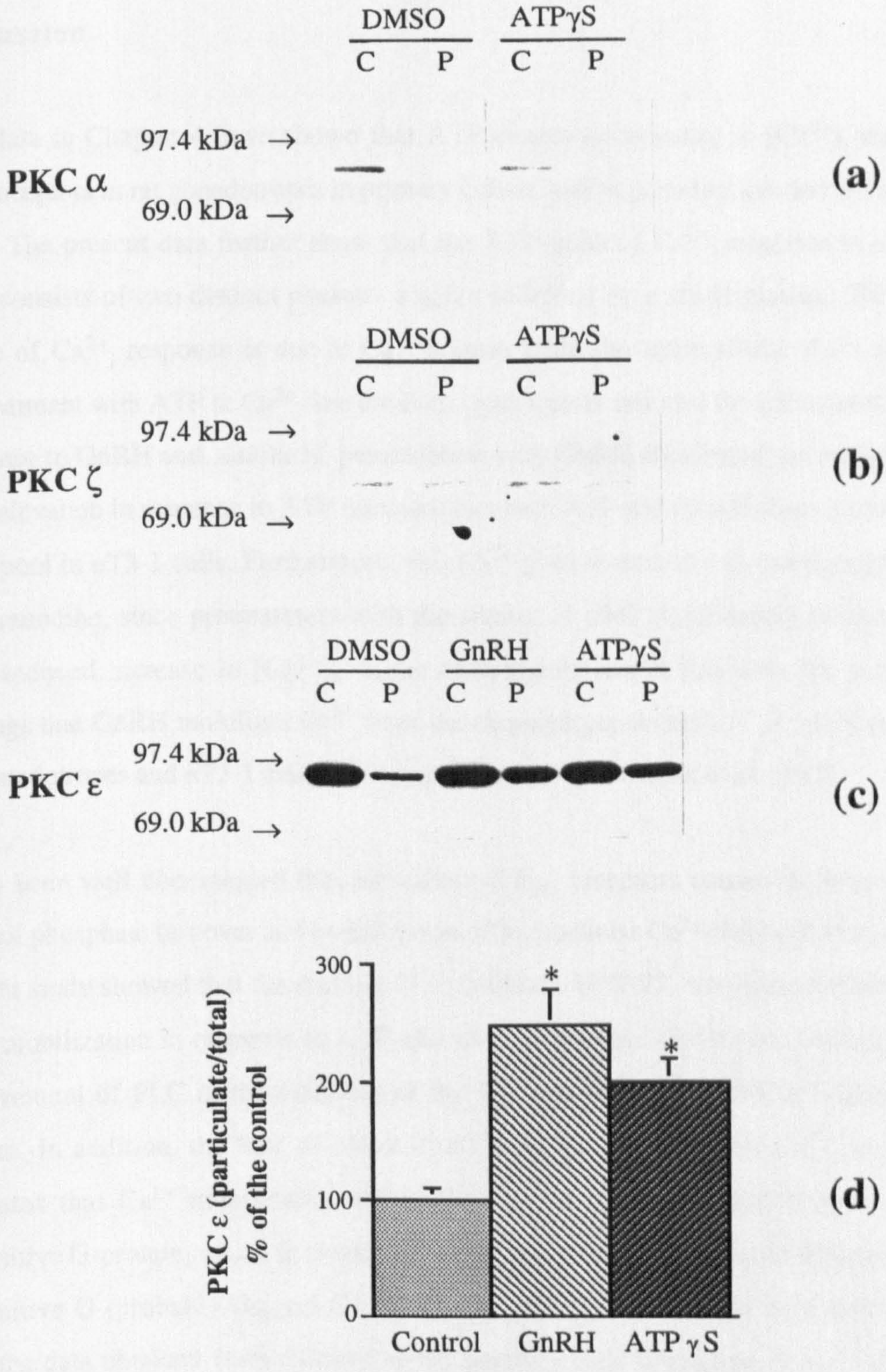


Figure 6-9. ATP receptor-mediated activation of PKC in αT3-1 cells. (a - c) Representative Western immunoblots show immunoreactivity of the cytosol (C) and particulate (P) fractions of αT3-1 cells with subtype-specific antibodies to PKCα, ζ and ε, respectively. (d) Histograms show the relative intensity of bands in the particulate fraction immunoreactive to the PKCε antibody. Values are mean ± SE of three separate experiments. *, $P < 0.01$ in comparison to the control. Cells were treated with stimuli for 10 min and the final concentrations of DMSO (as control), GnRH and ATPγS were 0.006%, 100 nM and 100 μM, respectively. 40 μg of total protein were loaded into each lane.

Discussion

The data in Chapter 4 have shown that ATP causes an increase in $[Ca^{2+}]_i$ via P_{2U} purinoceptors in rat gonadotropes in primary culture and in gonadotrope-derived αT3-1 cells. The present data further show that the ATP-induced Ca^{2+}_i response in αT3-1 cells consists of two distinct phases:- a spike followed by a small plateau. The first phase of Ca^{2+}_i response is due to Ca^{2+} release from the intracellular Ca^{2+} pools. Pretreatment with ATP in Ca^{2+}-free medium significantly reduced the subsequent Ca^{2+} response to GnRH and, similarly, pretreatment with GnRH eliminated the subsequent Ca^{2+} elevation in response to ATP indicates that both ATP and GnRH share a common Ca^{2+} pool in αT3-1 cells. Furthermore, this Ca^{2+} pool is sensitive to thapsigargin but not ryanodine, since pretreatment with the former (1 μM) significantly reduced the ATP-induced increase in $[Ca^{2+}]_i$. These observations are in line with the previous findings that GnRH mobilizes Ca^{2+} from the thapsigargin-sensitive Ca^{2+} pool in both rat gonadotropes and αT3-1 cells (McArdle & Poch 1992, Merelli *et al.* 1992).

It has been well documented that activation of P_{2U} receptors causes an increase in inositol phosphate turnover and mobilization of intracellular Ca^{2+} (Dubyak 1991). The present study showed that the specific PLC inhibitor, U73122, was able to inhibit the Ca^{2+} mobilization in response to ATP and, thus, provides a direct demonstration for involvement of PLC in the coupling of the P_{2U} receptor to the IP/Ca^{2+} signalling system. In addition, the lack of effect of PTX on the ATP-induced Ca^{2+}_i increase indicates that Ca^{2+} mobilization by ATP in αT3-1 cells is mediated by a PTX-insensitive G-protein, which is similar to GnRH receptors that are also linked to PTX-insensitive G (probably Gq and G11) (Hsieh & Martin 1992). This is in agreement with the data obtained from cultured sheep pituitary cells (Davidson *et al.* 1990) but differs from the findings from aortic endothelial cells, where P_{2U} receptors were found to be coupled to a PTX-sensitive G-protein (Motte *et al.* 1993).

Removal of extracellular Ca^{2+} has little effect upon the spike of ATP-induced Ca^{2+}

response but abolishes the second phase (plateau) of the response, indicating that Ca^{2+} influx is largely responsible for the plateau. The blockade of this Ca^{2+} influx by Cd^{2+} and nifedipine and by KCl-induced membrane depolarization suggests that during the plateau phase, ATP stimulates Ca^{2+} entry via voltage-sensitive Ca^{2+} channels. αT3-1 cells express both low and high voltage-gated Ca^{2+} channels (Bosma & Hille 1992) but the lack of effect of Ni^{2+} on Ca^{2+} elevation indicates the predominant involvement of a high voltage-sensitive Ca^{2+} channel in ATP-induced Ca^{2+} influx. The ATP-induced Ca^{2+} metabolism in αT3-1 cells, thus, resembles that provoked by GnRH, although the amplitude of an increase in $[Ca^{2+}]_i$ and, in particular, the plateau phase in response to ATP is smaller than that induced by GnRH (Merelli *et al.* 1992, McArdle *et al.* 1992). The mechanisms for triggering the Ca^{2+} influx, however, is not clear, although many theories have been proposed for the receptors which are coupled to IP/Ca^{2+} signalling, such as the capacitative model and receptor-activated Ca^{2+} influx (Putney Jr & Bird 1993).

In addition to the IP/Ca^{2+} signalling, G protein-linked ATP receptors have also been shown to utilize or interact with other signalling systems in some types of cell, which may occur directly or as a consequence of activation of the IP/Ca^{2+} system. Inhibition of agonist-stimulated accumulation of cAMP by ATP have been observed in NG108-15 neuroblastoma cells (Snider *et al.* 1984) and C6-2B glioma cells (Debernardi *et al.* 1991), and is thought to be caused by the increased cytosolic Ca^{2+}, which functions as a negative allosteric effector to reduce adenylyl cyclase activity (Steer & Levitzki 1975, Debernardi *et al.* 1993b). In other cells such as myocytes (Yamada *et al.* 1992), hepatocytes (Okajima *et al.* 1987), FRTL-5 thyroid cells (Okajima *et al.* 1989), renal LLC-PK1 cells (Anderson *et al.* 1991) and sertoli cells (Filippini *et al.* 1994), direct coupling of ATP receptors to adenylyl cyclase has been suggested for the inhibitory effect on cAMP generation. Apart from the inhibitory effect, ATP receptors might directly or indirectly increase cAMP production, as observed in aortic smooth muscle cells (Tada *et al.* 1992), Swiss 3T3 and 3T6 fibroblasts (Huang *et al.* 1991) and PTX-treated FRTL-5 thyroid cells (Sato *et al.* 1992). In addition to cAMP,

involvement of phospholipase D (PLD) and cGMP in the ATP receptor-coupled signal transduction has also been reported in some cell types (Snider *et al.* 1984, Martin & Michaelis 1989, Purkiss *et al.* 1993). However, the present data show that in αT3-1 cells activation of ATP receptors did not exhibit any significant effects on cAMP and cGMP generation and on forskolin- and PACAP38-induced cAMP accumulation and CNP-induced cGMP accumulation.

As ATP is capable of inducing inositol phosphate accumulation and an increase in $[Ca^{2+}]_i$ in many cell types, it has long been assumed that agonist occupancy of G protein-coupled ATP receptors, such as the P_{2U} subtype, would lead to activation of PKC. Such activation, however, has not yet been directly demonstrated (Boarder *et al* 1995). In the present experimental model using the Western immunoblotting with isozyme specific antibodies to examine PKC translocation in αT3-1 cells, we have been able to show that addition of ATPγS can cause modest but significant redistribution of PKCε. The present data hence has not only provided, to my knowledge, the first direct demonstration of PKC translocation by ATP receptors (P_{2U} subtype), but also implicate the potential importance of extracellular nucleotides in the regulation of gonadotrope function.

The PKC family consists of three groups:- conventional PKCs (cPKCα,βI, βII, and γ), which are activated by Ca^{2+}, diacylglycerol and phospholipid; novel PKCs (nPKCδ, ε, η, and θ), which are Ca^{2+}-independent and DAG-and phospholipid-activated enzymes; and atypical PKCs (aPKCζ and λ) (for review see (Nishizuka 1992)). Several isoforms of PKC (α, β, δ, ε and ζ) are present in rat pituitary cells (Garcia-Navarro *et al.* 1994) and PKCβII has been located in rat gonadotropes (Ohmichi *et al.* 1992). Activation of this enzyme family has been suggested to play mediatory roles in various cellular responses of gonadotropes (Counis & Jutisz 1991, Stojilkovic *et al.* 1994) although the precise roles have not been established in these cells (McArdle *et al.* 1987). In gonadotrope-derived αT3-1 cells, immunoreactivity to PKCα, ε and ζ (Johnson *et al.* 1993) and transcripts of PKCβ (Shraga-Levine *et al.* 1994) have been detected. GnRH

induces an increase in PKCβ gene expression in these cells (Shraga-Levine *et al.* 1994), but, surprisingly, only non-conventional PKCε and ζ but not conventional PKCα are translocated to the particulate fraction by GnRH (Kratzmeier *et al.* 1995). Similarly, the present data show that activation of ATP receptors also causes PKCε (but not α and ζ) translocation to the particulate fraction. The mechanism(s) underlying and the consequence of such translocation are, however, unknown at present. One feature of ATP receptors in αT3-1 cells is occurrence of rapid desensitization after agonist occupancy (Chen *et al.* 1994c) and this might be associated with activation of PKCε, as such activation has been shown to mediate feedback inhibition of PLC in rat basophilic RBL-2H3 cells (Ozawa *et al.* 1993).

Together, these data greatly strengthen the suggestion of a role for extracellular nucleotides in regulation or modulation of gonadotrope function.

Chapter 7. EXOCYTOTIC RELEASE OF ATP FROM PITUITARY CELLS

Introduction

The data described in previous chapters strongly suggest that pituitary ATP receptors may play an important role in the regulation of pituitary function. This suggestion raise the question of the source of extracellular nucleotides. It has long been known that the concentration of intracellular ATP is very high (at mM level) and that in many secretory cells, such as adrenal medulla cells (Smith 1968, Rojas *et al.* 1985), pancreatic ß-cells (Sussman & Leitner 1977), platelets (Born 1958), mast cells (Osipchuk & Cahalan 1992) and neurons (Fried 1980, Volknandt & Zimmermann 1986), secretory granules and vesicles contain ATP and other nucleotides in addition to specific transmitters and hormones. As far as pituitary cells were concerned, the local release of nucleotides may occur in conjunction with pituitary hormone secretion since the pituitary gland contains many secretory cells. The present study was to test this hypothesis by examining the real-time dynamic ATP release in an *in vitro* calcium ionophore-induced exocytosis model.

Methods

Pituitary glands from adult Wistar rats of both sexes were enzymatically dispersed and approximately 2 x 10^5 cells were plated out in a plastic vial with a 2 cm^2 surface area in M199 culture medium. After 2 days in culture, real-time dynamic bioluminescence measurements of ATP release on attached cells was performed as described (Chapter 2: page x). Under the present set-up condition, the light was linearly related to ATP concentrations in the reaction mixture between 5 x 10^{-12} and 5 x 10^{-8} M. Because ATP was determined by the bioluminescent method, a non-fluorescent form of calcium ionophore A23187, i.e. bromo-A23187 (Deber *et al.* 1985), was chosen to avoid

potential interference with the ATP assay.

Results

Addition of A23187 induced a significant release of ATP from cultured pituitary cells in a concentration-dependent manner, as indicated by increased luminescence (Figure 7-1). Compared to external ATP standards (0.005 nM - 10 nM), it was estimated that 20 μM A23187 induced the release of 3.15 ± 0.9 pmol of ATP (n = 5) from approximately 2×10^5 primary rat pituitary cells plated out 48 hr previously.

The simultaneous addition of 10 units of apyrase (an ATPase/ADPase for the reaction: $ATP \rightarrow AMP + 2P_i$) with A23187 abolished the increase in emitted light, and when added after A23187 rapidly reduced the A23187-induced light increase (Figure 7-2).

Removal of extracellular Ca^{2+} abolished A23187-induced ATP release but did not significantly affect the luciferase-luciferin reaction, since the application of external ATP still produced a comparable light signal to that in Ca^{2+}-containing medium (Figure 7-3).

Pretreatment with 20 μM A23187 for 28 min reduced the response to A23187 2 hours later by 80 ± 12.8 % (n = 4: Figure 4-4).

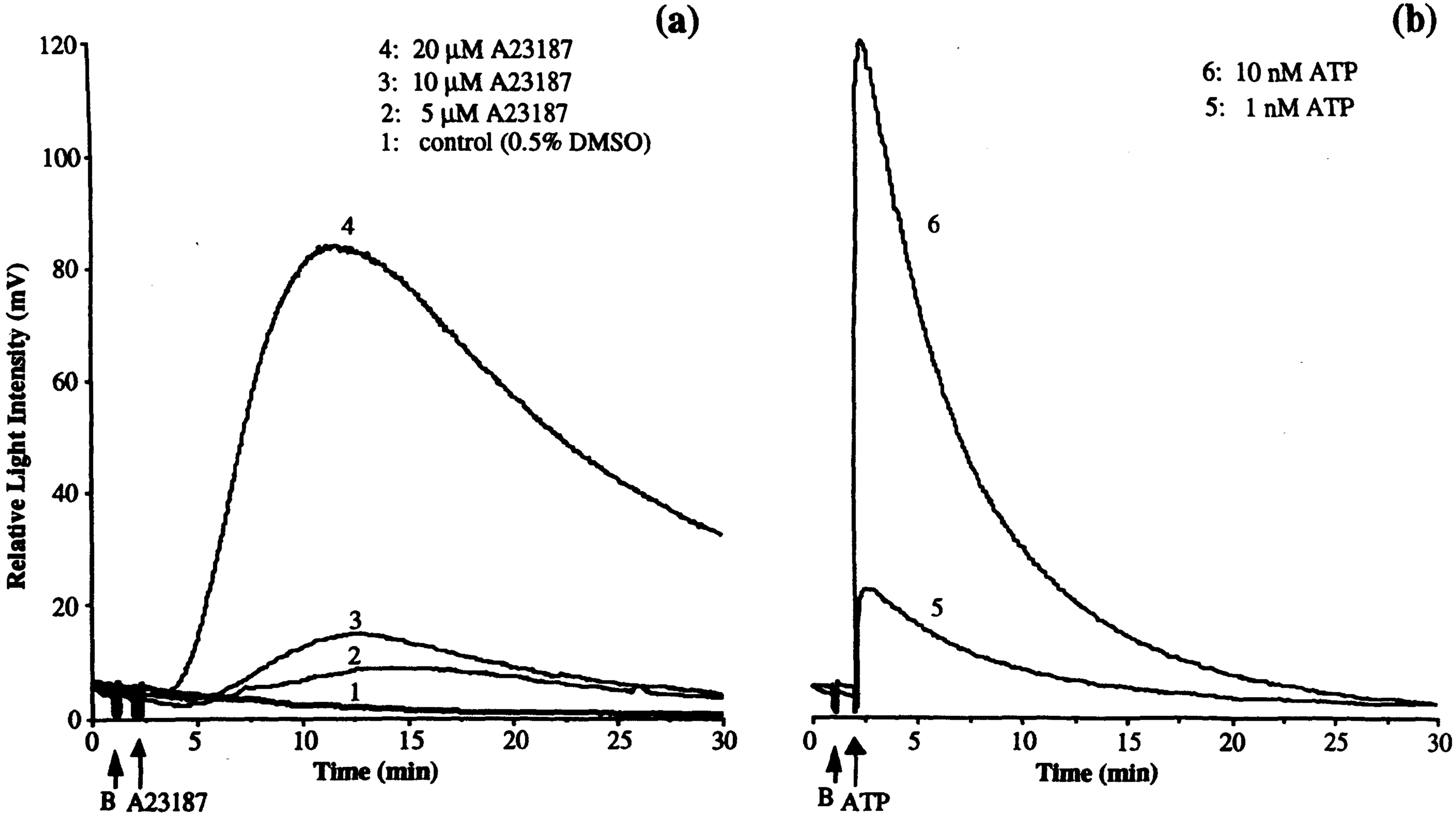


Figure 7-1. A23187-induced ATP release from primary pituitary cells in culture. (a) Representative recordings each from 4 separate experiments in which cells were exposed to A23187 (dissolved in DMSO and diluted in ATP assay buffer (final concentration of DMSO: < 0.5%)) following the addition of 10 µL ATP assay buffer (B). (b) Showing representative reference recordings each from 3 separate experiments using ATP standards externally added to cells. The addition of reagents are indicated by arrows and numbers (referred to their final concentrations).

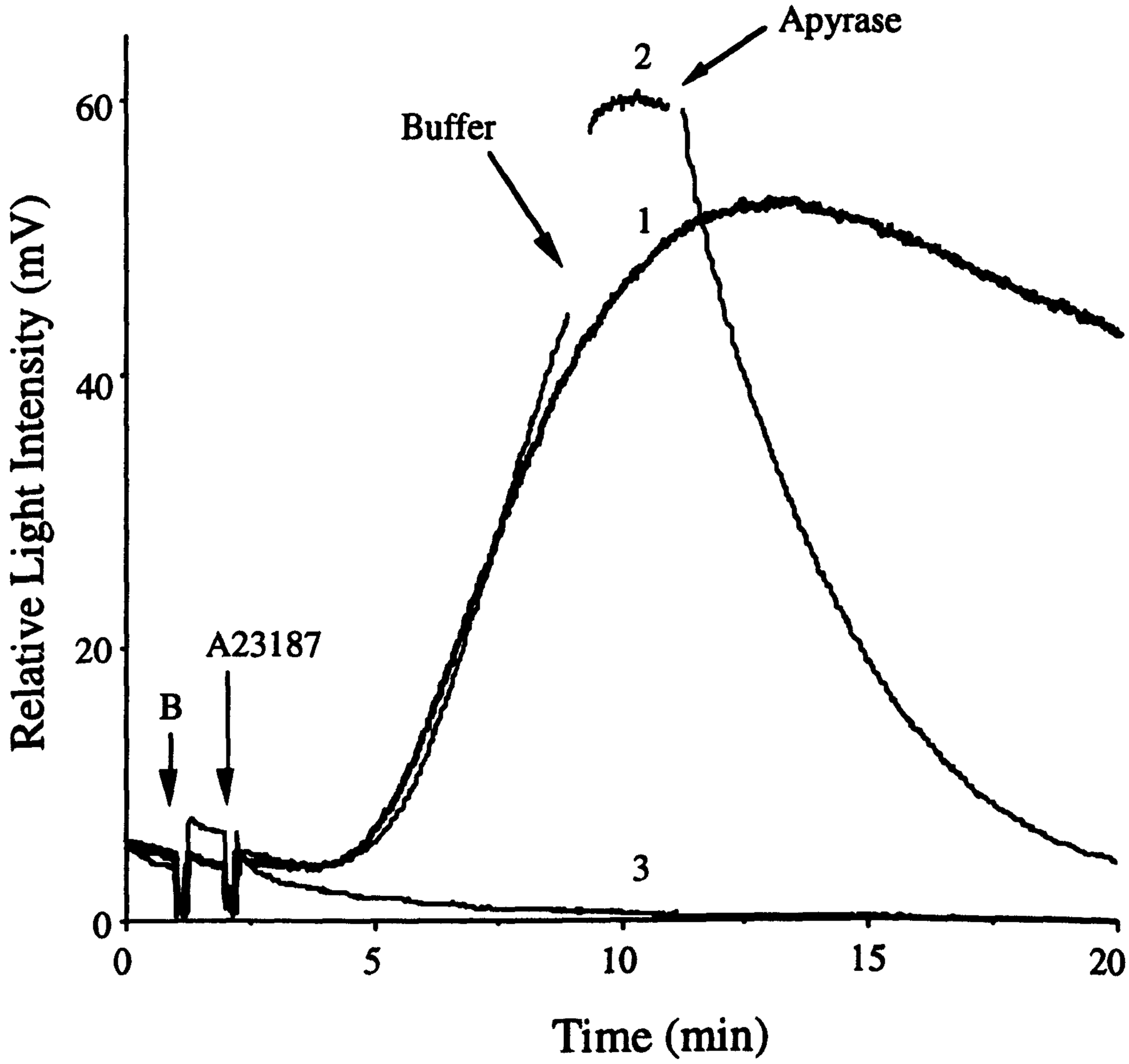


Figure 7-2. Depletion of extracellular ATP by apyrase. Following the addition of 10 µL of buffer (B), cells were stimulated with 10 µL A23187 alone (final concentration 20 µM: Trace 1) or A23187 as above plus 10 units of apyrase (in 6 µL: Trace 3). In another experiment, 6 µL of buffer and 10 units of apyrase (in 6 µL) were introduced sequentially to A23187-stimulated pituitary cells (Trace 2). Emitted light was not recorded during addition of reagents, hence the gaps in trace 2. Each trace shows representative recording from 3 separate experiments.

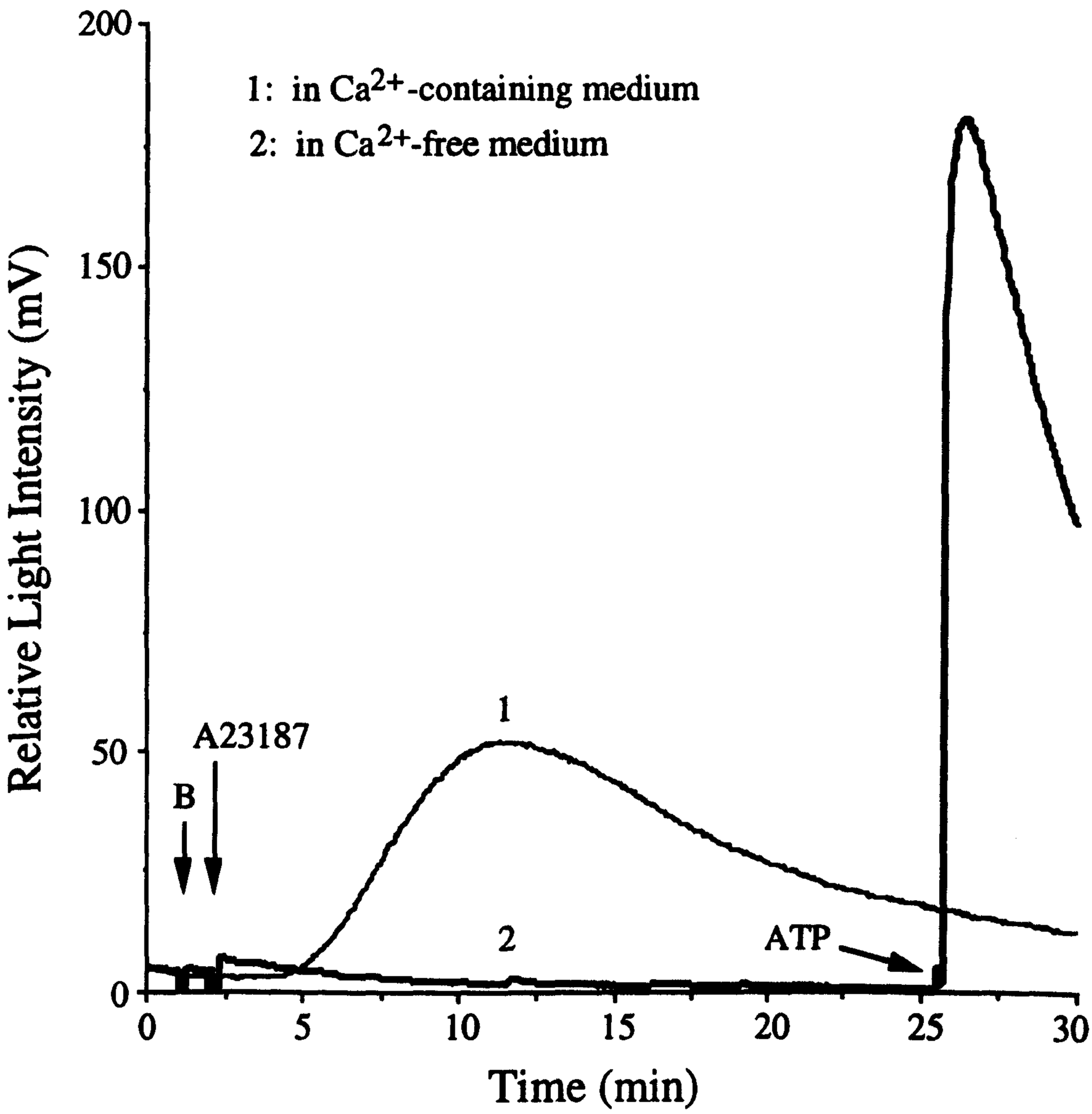


Figure 7-3. Dependency of A23187-induced ATP release on extracellular Ca^{2+}. Following the addition of 10 µL of buffer (B), cells were stimulated with A23187 (final concentration: 20 µM) in either Ca^{2+}-containing medium (4 mM: Trace 1) or Ca^{2+}-free medium containing 50 µM EGTA (Trace 2). The prompt response to the addition of external ATP in Ca^{2+}-free medium (final concentration: 20 nM) after 25 minutes (trace 2) confirmed the integrity of the assay system. Each trace shows representative recording from 4 separate experiments.

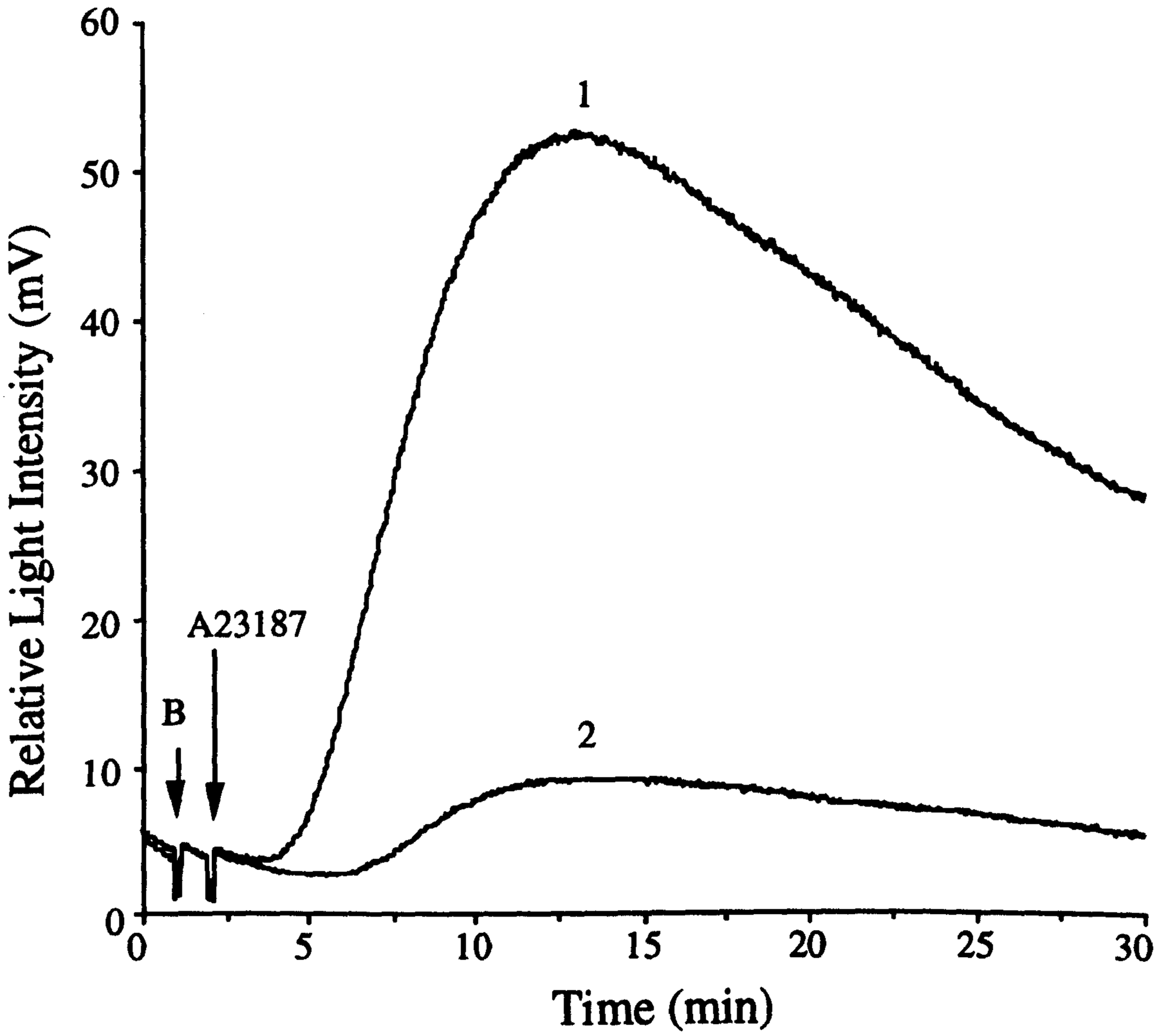


Figure 7-4. Desensitization of A23187 effect on ATP release. Following the addition of buffer (B), cells were stimulated with 10 µL of A23187 (final concentration: 20 µM: Trace 1), then thoroughly washed and incubated in cell culture medium containing 20 mM HEPES at 37°C for 2 hours. The identical procedure was then repeated on the same cells (Trace 2). Each trace shows representative recording from 4 separate experiments.

Discussion

The calcium ionophore-induced increase in light output in this study clearly demonstrates that substantial amount of ATP can be released from pituitary cells. The bioluminescence assay with luciferin and luciferase has been found very specific to ATP (Strehler & McElroy 1957, White 1978). The identification of ATP in the present experimental model is confirmed by the ability of apyrase to eliminate the increase in emitted luminescence (Figure 7-2).

As illustrated in Figure 7-1, the rate of increase in relative light intensity in response to A23187 was slower than that seen after the addition of exogenous ATP to the reaction mixture. These different profiles are likely to indicate occurrence of exocytotic secretion of ATP induced by the calcium ionophore. The exocytotic release of ATP is also implied by the data of Ca^{2+}-dependency (Figure 7-3) and the observed homologous desensitization (Figure 7-4). The latter presumably reflects depletion of secretory granule pools.

The obtained ATP release data imply a possible paracrine and/or autocrine mechanism by which the extracellular nucleotides may exert their effects on pituitary cells. Whether ATP is stored and released in its own right or co-stored and co-released with pituitary hormones is not known but the latter is favored by the findings from other cell types such as adrenal chromaffin cells (Douglas & Poisner 1966, Smith 1968) and pancreatic ß-cells (Sussman & Leitner 1977). The concentration of ATP in pituitary secretory granules and their possible co-existence with other nucleotides remains to be determined.

Chapter 8. OVERALL SUMMARY AND CONCLUDING REMARKS

ATP is well known for its metabolic function, namely, as the main chemical energy supplier for numerous cellular reactions. Work in the past two decades, however, has unveiled another important role for this molecule:- it is also a transmitter for communication between cells. By acting on its own large receptor family (P_2 purinoceptors), ATP has been implicated in a variety of biological processes including neurotransmission, cardiovascular function, platelet aggregation, secretion of insulin and surfactant, immune response and cell growth. The studies described in this thesis were undertaken to investigate a possible transmitter role for extracellular ATP in the neuroendocrine system.

By using calcium imaging at a single cell level, this study showed that exogenously applied ATP was able to provoke a rapid increase in $[Ca^{2+}]_i$ in a subset (~ 40%) of cultured rat hypothalamic neurons. This intracellular Ca^{2+} response was highly specific and mediated by ATP receptors of the P_{2X} subtype. Activation of these receptors resulted in influx of Ca^{2+} largely through high voltage-gated Ca^{2+} channels and ATP hydrolysis was not required for such activation. These findings indicate that ATP may act as an excitatory neurotransmitter to influence the hypothalamic function. Indeed, there is some *in vivo* evidence to support this. Adrenergic receptor antagonists by systemic and intracerebroventricular delivery or direct injection into the supraoptic nucleus (SON) failed to block A1-induced excitation of SON vasopressin cells (receiving a direct and excitatory noradrenergic input from the ventrolateral medulla A1 cell group) (Day *et al.* 1990), while local application of ATP and α,β-methylene ATP stimulated putative vasopressin cells an effect which was blocked by the ATP receptor antagonist suramin (Day *et al.* 1993). No electrophysiological recordings have been made in this study and therefore the nature of these ATP receptors, such as ion selectivity and channel kinetics, is still unknown. Furthermore the identity of ATP receptor-bearing cells and possible functional roles associated with ATP neurotransmission remain to be determined.

Previous work has shown that ATP and UTP, acting on "nucleotide receptors" (now called P_{2U} receptors), stimulate inositol phosphate accumulation and $^{45}Ca^{2+}$ efflux in mixed pituitary cell population {Davidson, 1990 #264}. The data from the current studies indicate that approximately one third of rat pituitary cells respond to ATP and that gonadotropes represent a specific population of ATP-targeting cells via a single class of ATP receptors characterized as the P_{2U} subtype. Owing to the small number of gonadotropes in the whole population of pituitary cells, it is very difficult to obtain relatively pure gonadotrope cell cultures. However, the gonadotrope-derived αT3-1 cells were also used in these studies and were shown to bear the P_{2U} receptors similar to those on gonadotropes, rendering an ideal alternative model to examine the signal transduction mechanisms subserving the gonadotrope ATP receptors. Experiments with these cells showed that activation of ATP receptors had no apparent effects on the cAMP and cGMP signalling systems but produced a biphasic cytosolic Ca^{2+} increase. The Ca^{2+} response was mediated by a pertussis toxin-insensitive and phospholipase C-coupled G-protein. Mobilization of intracellular Ca^{2+} from GnRH- and thapsigargin-sensitive Ca^{2+} pools and Ca^{2+} influx through high voltage-sensitive Ca^{2+} channels were responsible for the observed two phases of Ca^{2+} response.

Activation of G protein-coupled ATP receptors, such as the P_{2U} subtype, has been assumed to stimulate PKC but this assumption has not previously been directly demonstrated. Using Western immunoblotting to examine PKC translocation, I have been able to provide the first direct demonstration that agonist occupancy of G-protein linked ATP receptors can cause translocation of PKC (of the isozyme ε in αT3-1 cells).

At the 'physiological' level I have been able to link the intracellular Ca^{2+} response of gonadotropes to ATP with the secretory mechanism in these cells and have been able to demonstrate that extracellular ATP can achieve a significant release of luteinizing hormone from superfused rat pituitary cells. As gonadotropes only constitute a small proportion of pituitary cells (9% found in this study), the identification of other ATP-

responsive pituitary cells remains to be established. Some preliminary data obtained from the on-going study (not presented in this thesis) has shown that lactotropes are also direct target cells for ATP action.

The bioluminescence measurements with luciferin-luciferase revealed that a substantial amount of ATP (approximately 3 pmol of ATP released into a volume of 200 µl over 30 min by ~ 2 x 10^5 cells in response to 20 µM A23187) can be exocytotically released from pituitary cells in primary culture. Considering that the inter-cellular gaps in the intact tissue are very small, the high concentration (at the µM level) of ATP required for receptor activation could probably be achieved *in vivo*. How ATP is stored and released however has not yet been determined, but our data imply a possible paracrine and/or autocrine mechanism by which the extracellular nucleotides may exert their effects on pituitary cells.

There can be little doubt from the data described that ATP receptors play a role in pituitary function. Pituitary hormone secretion is principally regulated by classic hypothalamo-hypophyseal factors including GRH, somatostatin, GnRH, CRH, vasopressin, oxytocin, TRH and dopamine. Hence, the obvious question is to what extent ATP and other nucleotides, acting on ATP receptors, may exert physiological effects on pituitary hormone secretion *in vivo*. Though at present there is no experimental data available to address this question, it appears unlikely that nucleotides would act in the same dramatic fashion as do the classic hypothalamo-hypophyseal factors. It is possible and more likely, however, that these nucleotides may function in a subtle way so that many pituitary activities could be influenced or modulated. ATP might interact with hypothalamo-hypophyseal factors in regulation of hormone secretion. It might be utilized as an autocrine and/or paracrine factor for cells in the same and/or different population(s) for communication and modulation of cell activity. ATP might even affect pituitary cell growth and differentiation.

Overall, the studies in my dissertation strongly suggest that extracellular ATP and other

nucleotides play important roles in neuroendocrine function and suggest that further investigation into the identification of yet-unknown nucleotide-targeting cells could provide important functional data on the local regulation of neuroendocrine systems.

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